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Cytogenetic and RFLP analyses of somaclonal variation in Nicotiana tabacum

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CYTOGENETIC AND RFLP ANALYSES OF SOMACLONAL VARIATION IN *NICOTIANA TABACUM*

submitted by

Jane Sarah Cavell

for the degree of Ph.D.
of the University of Bath

1989

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'The time has come,' the Walrus said,
'To talk of many things:
Of shoes— and ships— and sealing wax—
Of cabbages— and kings'

Lewis Carroll

Abstract

Chromosome number variation occurred in regenerated *Nicotiana tabacum* plants. Aneuploidy at the amphidiploid level and mixoploidy had no readily detectable effects on phenotype, whereas an increase of chromosome number to or near to the tetraploid (2n=96) level was accompanied by characteristic morphological variation.

No variation occurred in the percentage of cultures that produced plants with high chromosome number between different explants and culture regimes, excepting a higher frequency of chromosome number variability in plants derived from leaf explants that had had their lower epidermis removed prior to culture. Different culture systems and explant types did, though, produce significant differences in the percentage of regenerated plants with high chromosome number. To test the hypothesis that chromosome number variation in regenerated plants derived from that present *in vivo*, Feulgen microdensitometry was used to estimate DNA content. The results showed that cells with DNA contents outside the normal, diploid range (2C-4C) were present in control leaf tissue.

Phenotypic variability was generally greater in plants regenerated from two-stage culture than in those from one-step culture, except for fasciation, the frequency of which was correlated with the number of plants produced per culture. Of 11 variable traits tested, one was transmitted to all progeny obtained by self-fertilisation.

The ability of restriction fragment length polymorphism analyses to detect genetic variation in different systems was examined. RFLP analyses successfully distinguished between different species of *Nicotiana* and between *N. tabacum* cultivars. In one regenerated plant out of 30, tested with 55 restriction enzyme/probe combinations, a change in band copy number was observed. No variation was detected between or within control seed-grown plants.

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Abbreviations and terminology used

A	adenine
Ap	ampicillin
ATP	adenosine triphosphate
BAP	6-benzylaminopurine
bp	base pairs of DNA
C	cytosine
°C	degrees centigrade
C-banding	visualisation of regions of constitutive heterochromatin
C-value	amount of DNA per haploid cell
cDNA	complementary DNA
Ci	Curie
CIM	callus induction medium
CMM	callus maintainance medium
CPW	cereal protoplast washing solution (see Appendix 1)
CTAB	cetyltrimethylammonium bromide
CTP	cytidine triphosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
epu	eye-piece unit (1 epu= 80μm)
g	gram
G	guanine
GTP	guanidine triphosphate
IAA	indole-3-acetic acid
k- (prefix)	kilo- ($\times 10^3$)
l	litre
LB	Luria broth
m- (prefix)	milli- ($\times 10^{-3}$)

m	metre
M	molar
MS	Murashige and Skoog medium (see Appendix 1)
n- (prefix)	nano- ($\times10^{-9}$)
n	haploid number of chromosomes
N	normal
OD	optical density
p- (prefix)	pico- ($\times10^{-12}$)
p	indicates a phosphate linkage between 2 bases
P	probability
rDNA	ribosomal DNA (i.e. the genes for ribosomal RNA)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecylsulphate
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume
2,4-D	2,4-dichlorophenoxyacetic acid
λ	bacteriophage lambda (or wavelength)
μ- (prefix)	micro- ($\times10^{-6}$)

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1. INTRODUCTION

Although tissue culture is widely used to propagate plants, a surprising number of the 'clones' produced are not identical to the parent plant. This phenomenon was recognised early in the history of plant tissue culture (see review by Dulieu (1972)) and has been called **somaclonal variation** by Larkin and Scowcroft (1981). It has been reported in a wide range of different species using many different explants and culture regimes, and many traits have been affected (reviewed by Scowcroft, 1985). Although high levels of genetic variability are undesirable in commercial micropropagation and germplasm storage programmes, somaclonal variation may not always be a disadvantage — there is great interest in exploiting it to develop new plant varieties:

1.1 APPLICATION OF SOMACLONAL VARIATION TO PLANT BREEDING

What can somaclonal variation achieve compared to existing plant breeding techniques? Culture-induced mutant phenotypes are often similar to known spontaneous mutations (Zehr *et al.*, 1986) and to physically and chemically induced mutations (Lee and Phillips, 1987). Sanford *et al.* (1984) remarked on the similarity between Russet Burbank protoclones and naturally occurring sports or 'bolters'. A comparison, however, of tomato somaclones with plants obtained following chemical mutagenesis showed that while most types of mutants were found in both classes, some mutants were only found among the somaclones (Gavazzi *et al.*, 1987). Other examples of novel mutants from tissue culture are given by Ahloowalia (1986).

One unusual feature of tissue culture derived mutants is that they are sometimes homozygous (Evans and Sharp, 1983; Lapitan *et al.*, 1984) and therefore true-breeding. Possible explanations for this include segregation of the mutation following mitotic crossing-over, or chromosome doubling after mutation in a haploid cell. Variation also occurs in characters thought to be under polygenic control, for example height and disease resistance (Larkin and Scowcroft, 1981). Somaclonal variation may thus be able to generate novel mutant types.

Conventional plant hybridisation strategies can only be applied to fertile plant species, whereas somaclonal variation can also be applied to sterile plants, or to highly heterozygous plants that do not breed true, and may thus become an important new tool for the improvement of vegetatively propagated crops such as potato. However, the range of plants that can be improved by somaclonal variation is limited because of the intractability of some plants *in vitro*.

Generally, somaclonal variation is not yet well enough understood for us to be able to control the types and frequencies of mutations that occur, so it does not have the advantages of a direct, defined mutagenesis system such as genetic engineering. This randomness usually leads to a requirement for large-scale screening for mutant isolation. In some cases, however, direct selection for particular mutants *is* possible, for example by growing cultures in the presence of salt, heavy metals or herbicides. This approach seems to be promising. For example, McHughen (1987) produced a stable line of flax plants that tolerated high soil salinity by including high salt concentrations in tissue culture growth media.

One drawback of somaclonal variation is that chromosomal abnormalities often occur in regenerated plants (D'Amato, 1985). Although this may not be a serious disadvantage in vegetatively propagated species, it may reduce the fertility and stability of new variants, so it is important that we learn how to control the amount of variation that occurs.

While somaclonal variation could be a useful addition to existing plant breeding techniques, then, the factors that control the nature and extent of variation must be analysed so that somaclonal variation can be exploited to its full potential.

1.2 DETECTION OF SOMACLONAL VARIATION

An essential part of the study of somaclonal variation is the development of techniques to monitor the occurrence of, and analyse the nature of genetic variation arising in tissue culture. Orton (1983) gives a critical review of experimental approaches that have been used to assess somaclonal variation. Many of these techniques have also been used to study genetic variability *in vivo*, for example, in pedigree analysis or in the assessment of evolutionary relatedness. Methods of detecting genetic variation, their uses and limitations are discussed below.

1.2.1 Measurement of phenotypic characteristics

While measurement of morphological characteristics can be helpful in monitoring genetic variation, it must be borne in mind that the phenotype is the result of an interaction between the genotype and the environment. Altered phenotypes may therefore be due to changes in gene expression as well as genetic change. For example, isozyme variation has been found between different tissues of a single plant (Arnison and Boll, 1975). It is therefore necessary to test the inheritance of a variant trait in order to show that it has a genetic basis. This approach is useful for the large-scale screening of regenerated plants. Since most mutations are recessive, and therefore masked in the heterozygous state, it is often more helpful to study the progeny of the regenerated plants when looking for mutant phenotypes.

The major drawback of phenotypic analysis is that it only screens that portion of the DNA that is expressed. A large proportion of higher plant DNA is never expressed, and much of the rest is not expressed at all times or in all parts of the plant. Even within genes, there are regions that are not transcribed or not translated, such as the 5' controlling sequences, and introns. It has also been pointed out by Beckmann and Soller (1986b) that many of the mutations in the third base of a codon will not alter the amino acid sequence, due to the degeneracy of the genetic code. A technique such as isozyme analysis is further limited since it can only detect those changes which cause a net change in the charge of the protein (Brown and Weir, 1983). In conclusion, then, phenotypic analysis can only detect a small proportion of genetic variation. Studies of the genome itself should give a more representative picture of the extent of genetic variation.

1.2.2 Cytogenetic analysis

Chromosome counting is frequently used as a measure of genetic variation, but has several drawbacks: it becomes more difficult as chromosomes become smaller and more numerous; it is limited to those cells that are dividing, and the genetic status of these dividing cells may not be representative of the entire population of cells; chemicals such as colchicine and 8-hydroxyquinoline used to improve chromosome preparations can induce polyploidy and therefore produce misleading results.

Chromosomal structural changes can be inferred from mitotic and meiotic analysis and from chromosome banding techniques (Schulz-Schaeffer, 1980), but the range of plants to which such techniques can be applied is limited. A useful combined technique is the hybridisation of DNA probes directly to chromosome spreads: this '*in situ* hybridisation' can reveal chromosome abnormalities.

1.2.3 DNA content estimation

Cellular DNA content can be estimated by measuring the intensity of Feulgen staining using a microdensitometer. Sampling is relatively easy, and the main advantage is that cells at any stage of the cell cycle can be used. However, there is a high error component associated with this method, (Orton, 1983), making it suitable only for the detection of polyploidisation and not for precise determination of chromosome number.

1.2.4 Physical properties of DNA

The kinetics of DNA melting and renaturation depend on the proportions of different sequence classes and can therefore be used to analyse the overall composition of the genome (Flavell, 1982). Studies of reassociation between DNA from two different sources can be used to measure sequence homology. Such studies are widely used to assess the composition of the genome in terms of sequence repetitivity, and to measure the relatedness of different species.

Base mismatches in a heteroduplex (i.e. an artificial double-stranded DNA molecule formed from 2 non-identical strands) can also affect the electrophoretic mobility of the DNA in denaturing gels; this phenomenon has been exploited by Myers *et al.* (1985) to detect single base substitutions. However, such analyses require that the 'normal' nucleotide sequence is known, and that the region to be tested is cloned for each sample; this technique is not, therefore, suitable for large-scale screening.

The base composition of DNA determines its buoyant density and thus DNAs of significantly different base composition can be separated by density gradient ultracentrifugation (Flavell, 1982). Qualitative and quantitative changes in 'satellite' (i.e. secondary) bands on a gradient may indicate gross genomic changes, but it is important to bear in mind that methylation of DNA (and therefore epigenetic change) can affect buoyant density (Kemp and Sutton, 1975).

1.2.5 High resolution analysis using molecular genetics

The ultimate way of analysing DNA is to determine its base sequence. Although sequencing is laborious and impractical for screening large numbers of samples, it is a powerful method for determining the nature of genetic variation. Generally, restriction digestion is a more appropriate method for the routine monitoring of genetic variation. Restriction enzymes recognise and cut at specific base sequences in DNA; a given piece of DNA will thus be cut into a characteristic set of fragments, which can be separated by gel electrophoresis. Mutations can lead to changes in fragment length (**restriction fragment length polymorphisms**, or RFLPs) or intensity (see Figure 1).

Restriction digests of DNA from small genomes such as mitochondrial or chloroplast DNA are simple enough to examine directly. Eukaryotic nuclear genomes, however, are very large, and therefore when digested with restriction enzymes, they produce very many bands, which appear as a continuous smear. Only highly repeated sequences are visible as discrete bands. The whole genome cannot be studied at once: instead, a subset of the bands present in the smear can be picked out by hybridising the DNA with a radioactively labelled DNA sequence or 'probe'. The band pattern obtained, and the frequency of RFLPs detected, will depend on both the enzymes used to digest the DNA, and on the nature of the probe. These factors are discussed in more detail in Section 4.1. The amount of polymorphism detected is also species-dependent. For example, maize is highly polymorphic — most probe/enzyme combinations tested by Helentjaris *et al.* (1985) detected polymorphisms between inbred lines. Much less variation was detected by the same authors in tomato. Pea, beans and sunflower also showed less variation (Helentjaris and Blake, unpublished results cit. in Beckmann and Soller, 1986b).

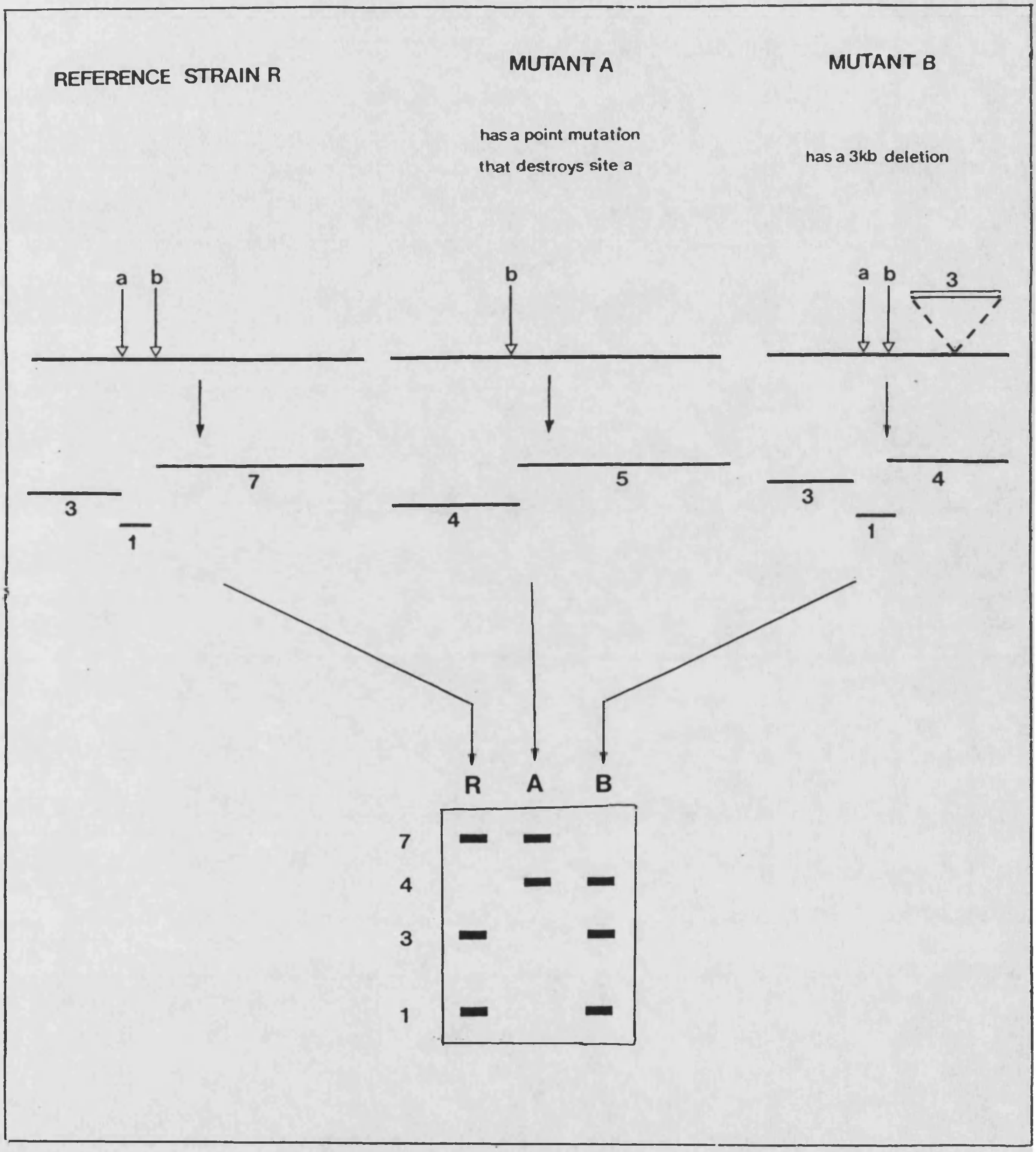
RFLPs enable the analysis of regions of the genome that are inaccessible by other methods. Bunce *et al.* (1986) used a hordein cDNA clone to distinguish different cultivars of barley that had indistinguishable hordein gel electrophoresis patterns. Another advantage of RFLPs (and of biochemical markers such as isoenzymes) is that they behave codominantly, that is, both alleles at a locus can be detected. Since they study the DNA itself, any tissue at any stage of the life cycle could be used for DNA extraction.

One disadvantage of RFLPs is that only a tiny fraction of the genome can be studied at once. The amount of DNA surveyed, and the likelihood of detecting variation, can be increased by using so-called 'fingerprinting' probes that hybridise to multiple, hypervariable loci. Such probes, developed by Jeffreys *et al.* (1985), have been successfully used to distinguish individual humans and other vertebrates. Recently, the original ' Jeffrey's probe' was used to identify different cultivars of rice, but individual plants could not be differentiated (Dallas, 1988). Part of the M13 bacteriophage

facing page 6

Figure 1: The effects of mutations on restriction digestion patterns

The DNA of a hypothetical reference strain, R, is cleaved by a restriction enzyme, yielding three fragments. In mutant A, a point mutation has destroyed site a; mutant B has a deletion. Such mutations can lead to altered restriction sites (top), thus giving altered restriction fragment sizes (centre) which can be detected by gel electrophoresis (below)


REFERENCE STRAIN R
MUTANT A
has a point mutation
that destroys site a
MUTANT B
has a 3kb deletion
a
b
b
a
b
3
7
3
1
5
4
3
4
1
R
A
B
7
4
3
1

genome was fortuitously found to hybridise to human minisatellite DNA (Vassart *et al.*, 1987) and has also been tested on a range of plant species (Rogstad *et al.*, 1988): individual plants could be distinguished in some species, including several angiosperms (e.g. *Populus deltoides*) and a gymnosperm (*Pinus torreyana*) but other species, such as tomato, were very uniform.

Although RFLPs are used to examine the DNA itself, the patterns obtained can sometimes be influenced by non-genetic factors. For example, some restriction enzymes will not cut at their recognition sequence if particular bases are methylated (reviewed by Nelson and McClelland, 1989). Changes in methylation, which can be associated with changes in gene expression, and which have been found in cultured cells of melon (Grisvard, 1985), soya bean (Quemada, 1987) and carrot (LoSchiavo *et al.*, 1989), could therefore alter restriction patterns. It is thus important that enzymes of known methylation sensitivity are used: this information is available for many enzymes and is updated frequently (e.g. Nelson and McClelland, 1989). Also, some restriction enzymes show 'star activity' (that is, they become able to cut at additional sequences), in suboptimal reaction conditions (Fuchs and Blakesley, 1983), so it is important to use the correct buffer consistently.

1.3 THE NATURE OF SOMACLONAL VARIATION

Many genetic changes have been found in cultured cells and regenerated plants using the techniques described above. Examples are discussed below:

1.3.1 Single gene changes

Detailed genetic analysis to confirm a single gene trait has been completed by Evans and Sharp (1983, 1986) for several tomato somaclonal variants. Most of the mutations were recessive and heterozygous, but dominant and homozygous mutations were also found. Other examples of mutations with segregation frequencies suggesting classical 'single-gene' mutations have been reported in *Nicotiana* (Prat, 1983), soya bean (Barwale and Widholm, 1987), maize (Lee and Phillips, 1987) and rice (Fukui, 1983) among others. A number of people have studied mutations at known marker loci. Barbier and Dulieu (1980) regenerated plants from protoplasts of *Nicotiana tabacum* plants heterozygous at two loci; both homozygous mutant and homozygous wild-type plants were obtained. A similar system was used by Lörz and Scowcroft (1983).

1.3.2 Chromosomal abnormalities

There is now much literature about the occurrence of chromosomally variant cells in culture. Polyploidy, aneuploidy, chromosome structural changes and mitotic abnormalities have been reported in tissue cultures of a wide range of species (reviewed by Bayliss, 1980). The extent of chromosomal variation in regenerated plants is generally less than that in culture, suggesting that many chromosomal abnormalities prevent regeneration. Nevertheless, both numerical and structural chromosome variation in regenerated plants have been reported frequently. This subject is comprehensively reviewed by D'Amato (1985).

Chromosomal abnormality in cultured cells can be extensive: for example, Gould (1982) concluded that gross chromosomal rearrangements in *Brachycome dichromosomatica* suspension cultures could produce karyotypes in which *all* the chromosomes had altered C-banding patterns. The most common cytogenetic alteration in plants regenerated from oat tissue culture was chromosome breakage followed by partial chromosome loss (McCoy *et al.*, 1982): this occurred in over 20% of regenerated plants. Trisomy, monosomy, interchanges and inversions also occurred. Lapitan *et al.* (1984) studied the C-banding of chromosomes in regenerated wheat × rye hybrids and found that small deletions were the most common change. Their work also showed that particular regions of the genome may be more susceptible to change than others: 12 of the 13 chromosome breakpoints involved in translocations and deletions were in the heterochromatin.

why do you suspect this?

Many genetic changes may be due to change at the chromosomal level. Of 551 regenerants of hexaploid wheat, 17 had variant alcohol dehydrogenase (ADH) isozyme patterns, and 13 of these were attributed to aneuploidy for the chromosome carrying the *Adh* locus (Davies *et al.*, 1986). Peerbolte *et al.* (1987) looked at the T-DNA of a 'shooty' crown gall line; one plant reverted from the normal non-rooting, octopine-synthesising traits, although it retained other traits encoded by the T-DNA. Southern blots indicated that deletion, and possibly amplification had occurred.

1.3.3 Variation in DNA content

DNA content changes have been reported in regenerated plants, for example, in protoplast-derived potato plants (Sree Ramulu *et al.*, 1984), but such data is difficult to interpret, since the errors in the techniques used do not allow precise determination of DNA content. Some apparent cases of DNA content variability in tissue culture-derived plants may be due to the reduced stainability of tissues containing polyphenols (Greilhuber, 1988). This is a particular problem with genera such as *Pinus*. It can be avoided by using formaldehyde rather than methanol/acetic acid as a fixative.

1.3.4 Single base changes

A total of 1382 plants regenerated from maize immature embryos were screened for changes in ADH isozyme patterns; the nature of two variants obtained was investigated by sequencing the entire *Adh* gene. Both plants had a single base change, one resulting in an altered amino acid which explained the altered electrophoretic mobility (Brettell *et al.*, 1986a) and the other resulting in the creation of a new stop codon, causing complete loss of the ADH protein (Dennis *et al.*, 1987). A triazine resistant *Nicotiana plumbaginifolia* mutant isolated from cell culture was found to have two single base changes in the *psb*A gene in the chloroplast DNA (Páy *et al.*, 1988).

1.3.5 Amplification and deamplification

Many different techniques have shown that the relative proportions of different parts of the genome can alter in cultured cells and regenerated plants. The early literature is reviewed by Buiatti (1977). De Paepe *et al.* (1982) found an increased percentage of highly repeated DNA in tobacco doubled haploids using reassociation kinetics, and also reported an increased proportion of a G+C rich satellite band on a caesium chloride gradient. A similar phenomenon was reported in *Vicia faba* root dedifferentiation by Natali *et al.* (1986). Changes in both G+C and A+T rich fractions of the DNA were reported in the first few hours of *Nicotiana glauca* pith culture (Durante *et al.*, 1983). Hase *et al.* (1979, 1982), though, found that an apparent increase in G+C rich satellite during carrot cell dedifferentiation was due to earlier replication of this DNA compared to the main band DNA. It is also important to bear in mind that changes in the methylation of satellite DNA, which do occur in culture (Grisvard, 1985) could cause misleading changes in the physical properties of the DNA (Kemp and Sutton, 1975).

More conclusive evidence for amplification comes from molecular genetics and cytology. Brettell *et al.* (1986b) used Southern blotting to show that one of 192 *Triticale* plants regenerated from immature embryos had a significant reduction in the amount of ribosomal DNA (rDNA) spacer sequences. C-banding of the chromosomes showed much reduced staining at one of the *Nor* (nucleolar organiser) regions. The deamplification was inherited.† Deamplification of rDNA was also found in potato protoclones (Landsmann and Uhrig, 1985) and in flax callus (Cullis and Cleary, 1986a).

Tandemly repeated sequences may be particularly prone to changes in copy number: only one of nine probes for tandemly repeated sequences failed to show copy number changes between flax leaves and leaf-derived callus (Cullis and Cleary, 1986a). Lapitan *et al.* (1988) detected heritable amplification of the same 480bp sequence in two *Triticale* regenerants using *in situ* hybridisation, suggesting that this region is particularly prone to change.

Amplification of particular sequences can occur in direct response to selection. A well-known example from animal cells is an increase in the number of copies of the dihydrofolate reductase gene following methotrexate resistance (Schimke *et al.*, 1988). A similar phenomenon has been reported in alfalfa suspension cultures (Donn *et al.*, 1984) which responded to the addition of the glutamine synthetase (GS) inhibitor, L-phosphinothricin, by increasing the GS gene copy number. The change, which was detected by Southern blotting, was maintained for a year in the absence of L-PPT, suggesting that the amplified sequences were integrated into the genome. A herbicide resistant *Petunia hybrida* cell line was obtained by selection of cultures on glyphosate: the resistant cells had an increased level of the herbicide's target enzyme. The mechanism of this amplification was not determined (Steinrücken *et al.*, 1986).

1.3.6 Transposition

Some types of mutations found *in vivo* are particularly unstable, with frequent reversions to the non-mutant phenotype. Most of such mutations are thought to result from transposition, that is the insertion (or excision) of a transposable element, causing inactivation (or reactivation) of the gene (Döring and Starlinger, 1986).

The high frequency of reversion of a white-flowered somaclonal variant of alfalfa to the normal, purple colour (Groose and Bingham, 1986a and b) could be due to transposition; however, a high

† Recently, Breiman *et al.*, (1989) revised their interpretation of *Nor* region variability in regenerated wheat plants; they found that variation exists between grains in a single spike of a seed-grown plant. It is therefore necessary to treat other reports of *Nor* region variability with some caution.

reversion frequency is not, in itself, proof of transposable element activity.

In vivo, activation of transposable elements may be associated with chromosome breakage (McClintock, 1984). The similarity of the patterns of chromosome breakage occurring in cultured cells and regenerated plants with those associated with transposition suggests that transposition does occur *in vitro* (Dellaporta and Chomet, 1985, Lee and Phillips, 1988).

Only a few transposable element systems have been well characterised; most of these are in maize (Döring and Starlinger, 1986). Studies of the maize transposable element *Ac* showed that this element was activated in some maize somaclones (Peshke *et al.*, 1987) but data about other species are lacking due to the limited range of plant species in which transposons have been isolated.

Molecular evidence for transposition in tissue culture is limited due to the scarcity of molecular probes for transposons. Recently, though, an elegant study by Grandbastien *et al.* (1989) demonstrated that three independent nitrate reductase deficient *Nicotiana tabacum* mutants derived from protoplasts contained a 5.5kb insertion at the nitrate reductase locus; the inserted sequence, which was in a different part of the gene in each case, was sequenced and found to have structural similarities to retroviral-like transposable elements.

1.3.7 Organellar genome variation

Maternal inheritance of a mutation suggests that the alteration has occurred in the chloroplast or mitochondrial DNA. This phenomenon has been observed in regenerated plants in several characteristics, including male sterility in *Nicotiana sylvestris* protoclones (Li *et al.*, 1988) and triazine resistance in *Nicotiana* regenerants (Csélpó *et al.*, 1985).

Since organellar genomes are small enough to allow direct examination of restriction fragments, the stability of organellar DNA has often been measured by looking for RFLPs. There are many examples of variation in mitochondrial DNA in cultured cells and in regenerated plants. The most frequent change observed has been an alteration in the relative stoichiometry of restriction fragments but these are difficult to interpret in the light of the findings of Borck and Walbot (1982) who concluded that the nuclear background could influence the relative stoichiometry of mitochondrial DNA restriction fragments. Both quantitative and qualitative changes were found in the mitochondrial DNA of 7 out of 47 potato protoclones (Kemble and Shepard, 1984). Similar results were observed by Negruk *et al.* (1986) in *Vicia faba* suspension cultures: these authors also reported an increase in the heterogeneity of the sizes of the circular mitochondrial DNAs in the cultured cells compared to seedlings. These changes may reflect the capacity of the mitochondrial DNA to recombine, leading to genome reorganisation, which does not necessarily result in a change in information content (Mulligan and Walbot, 1986).

Chloroplast DNA, in contrast, seems to be remarkably stable *in vitro*. No chloroplast DNA changes were found in 10-year-old carrot cell cultures (Matthews and DeBonte, 1985) or in potato protoclones (Kemble and Shepard, 1984). This stability is also observed *in vivo*: the chloroplast DNA is very highly conserved both within and between species. For example, Palmer and Thompson (1982) found only minor variations in chloroplast genome organisation between mung bean, spinach, cucumber, *Petunia*, and maize. One exception to this general rule of chloroplast stability is the high frequency of chloroplast DNA deletions found in anther culture derived plants (Day and Ellis, 1984).

1.4 ORIGIN OF SOMACLONAL VARIATION

There is evidence that some genetic variability can exist *within the explant* itself, and that other variation can arise as a result of tissue culture:

1.4.1 Genetic variability exists within a plant

1.4.1.1 Somatic mutation

Most naturally occurring mutations are thought to arise from errors in the DNA replication process (Watson *et al.*, 1987) and so during the many cell divisions that produce a mature plant, some mistakes must arise, even when the mutation rate is low (Klekowski, 1988). A mutation occurring early on in development could be passed on to many daughter cells, leading to a whole mutant sector within a plant.

Many of the known examples of somatic mutation are from horticulture, where 'sports' have been derived spontaneously from existing varieties. For example, in the potato cultivar Russet Burbank, sports occur at a high frequency in the field (Sanford *et al.* (1984)), suggesting that the level of within-plant variation can be high. The frequency of production of 'rogue' plants, that is, those with undesirable phenotypes, may be particularly high — up to 1% of the population — in some vegetable crops, including lettuce and the garden pea (Pearson,1968), and it has been suggested that this instability is associated with transposable element activity.

One special case of within-plant variation is that of chimaeras. These will obviously segregate into different cell types *in vitro*. For example, some plants regenerated from callus from the chimaeric *Pelargonium* variety, 'Rober's Lemon Rose', showed reversion to the non-chimaeric, parental leaf forms (Skirvin and Janick, 1976). The same authors showed that the variegated *Pelargonium* variety, 'Snowflake', was also unstable *in vitro*.

1.4.1.2 Somatic crossing-over

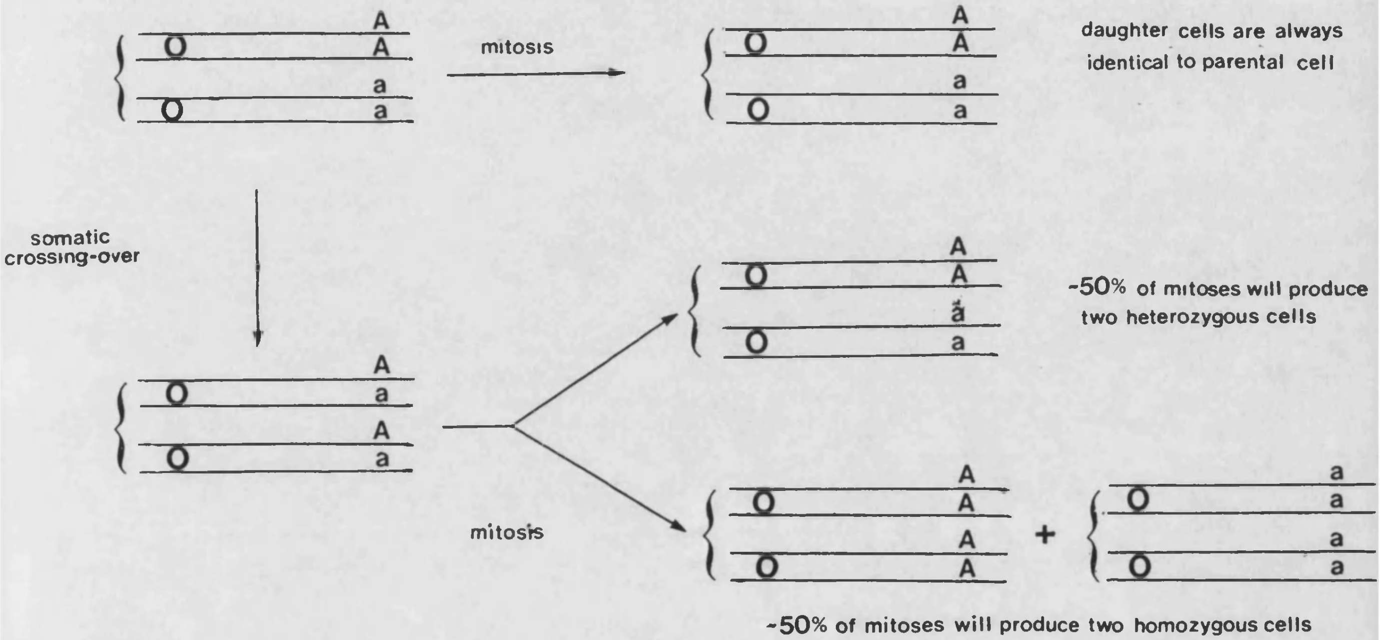
Normally, the two chromatids of one chromosome are identical, so all daughter cells produced by mitosis are identical. However, if mitotic crossing-over occurs between two homologous chromosomes at a heterozygous locus, chromosomes with non-identical chromatids can be produced. This can lead to segregation in the daughter cells (see Figure 2). This 'somatic crossing-over' can be detected by the appearance of spots with the homozygous mutant or homozygous wild-type phenotypes in a heterozygous plant. The frequency of somatic crossing-over has been estimated at 7.70×10^{-6} to 5.74×10^{-5} per locus per mitosis (Evans and Paddock, 1976). An important consequence of somatic crossing-over is that a single cell could produce daughter cells of two different genotypes, as illustrated in Figure 2. Genetic variation among the plants derived from a single protoplast is therefore *not* necessarily proof of culture-induced mutation, but may be merely expression of an event that occurred *in vivo* prior to culture initiation.

1.4.1.3 Chromosomal variation in vivo

Some genetic change may take place *in vivo* as a normal part of development. There are many examples of polyploid tissues occurring within normally diploid plants (D'Amato, 1977); such **endopolyploidy** can contribute to the chromosomal heterogeneity found *in vitro*. For example, Murashige and Nakano (1967) found an increased level of polyploidy in older stem pith of tobacco compared to younger regions, and a corresponding increase in the frequency of polyploid cells in callus cultures initiated from the older tissue. The presence within one plant of some cells with

Figure 2: Consequences of somatic crossing-over at a heterozygous locus

Above: in normal circumstances, each chromosome has two identical chromatids, so mitosis yields cells identical to the parent cell, i.e. heterozygous at this locus.
Below: if somatic crossing-over has occurred, each chromosome will have one of each allele. Segregation of the chromatids at mitosis will, about 50% of the time, produce two cells homozygous at this locus.


O A A O a a
mitosis
daughter cells are always identical to parental cell
somatic crossing-over
O A a O A a
-50% of mitoses will produce two heterozygous cells
mitosis
+
-50% of mitoses will produce two homozygous cells

aneuploid chromosome numbers or with structural chromosomal changes has also been reported. For example, chromosome number mosaicism has been found in sugarcane (*Saccharum officinarum*) (Nickell, 1977). Anueploidy and polyploidy occurred in plants regenerated from cultures of tetraploid alfalfa, but comparable levels of variation were observed in control seed-grown plants (Feyer *et al.*, 1989); these findings suggest that alfalfa may be chromosomally mosaic. Torrey (1965) initiated callus from pea root segments in the presence of tritiated thymidine. He observed numerous tetraploid mitoses with ***unlabelled*** diplochromosomes. These results suggest that DNA synthesis had occurred prior to callus induction, and that kinetin stimulated mitosis and cell division in these endoreduplicated cells.

1.4.1.4 DNA content changes in vivo

Altamura *et al.* (1987) showed that DNA from different regions of the tobacco stem had different thermal denaturation properties, suggesting that the G+C content of the DNA varied within the plant. This indicates that amplification or deamplification of specific parts of the genome may occur *in vivo* , but differences in methylation, and their effects on thermal denaturation, were not investigated.

In the development of some animals, for example the toad, *Xenopus laevis*, a temporary massive demand for rRNA is met by the extrachromosomal amplification of rRNA genes (Gall, 1968); variation in the copy number of rRNA genes has also been found within individual plants in flax (Cullis and Charlton, 1981) and in *Vicia faba* (Rogers and Bendich, 1987), but in neither case was the change tissue-specific. The functional significance of the amplification in plants, if any, is not known.

Fragmentation of DNA may occur as a part of the ageing process. For example, Chen and Srivastava (1986) reported a progressive decrease in the mean size of restriction fragments from barley leaves of increasing age.

From these examples, it is clear that the original explant is not necessarily genetically uniform. The extent of variation present will depend on many factors such as species and culture system. These factors are discussed in more detail in Section 1.5.1.

1.4.2 Genetic variation can arise in culture

1.4.2.1 Evidence

Both chromosomal variation (Karp *et al.*, 1982) and genic changes (Lörz and Scowcroft, 1983) have been found between plants derived from a single protoplast. Although the former is suggestive of culture-induced variation, the latter could be explained by somatic crossing-over having occurred *in vivo*, and then being expressed by cell division in culture (see Figure 2).

Chromosomal abnormalities are also found in cultures from tissues that are thought to be chromosomally uniform *in vivo*, such as those from non-polysomatic plants (Sacristán, 1971) and meristems (Natali and Cavallini, 1987). Singh (1986) found that embryo-derived callus of barley was initially 100% diploid, but became gradually more karyologically abnormal with time. Mitotic abnormalities seen *in vitro*, such as multipolar anaphases and lagging chromosomes, may be responsible for at least some of the chromosomal abnormalities found in cultured cells.

An increasing number of mutations with length of time in culture (e.g. Orton, 1985; Lörz and Scowcroft, 1983) also suggests that mutations do occur *in vitro*; the sequential occurrence of mutations has been demonstrated by pedigree analyses such as those of Fukui (1983) and Benzion and Phillips (1988).

1.4.2.2 Causes of culture-induced variation

1.4.2.2.1 Are culture components directly mutagenic?

Reports of the effects of hormones on somaclonal variation give conflicting evidence (Bayliss, 1980), probably because it is difficult to determine whether an apparent increase in variation is due directly to mutagenesis or whether a particular combination of hormones selects for the growth of specific cell lines and thus indirectly alters the proportions of different cell types.

No mutagenic effect was observed for IAA, 2,4-D or kinetin on the highly mutagen sensitive *Tradescantia* stamen hair colour system (Dolezel and Novák, 1984). The increased frequency of sister chromatid exchange found in garlic cell cultures in the presence of 2,4-D (Dolezel *et al.*, 1987) could indicate that this hormone is mutagenic, but could also be explained by the lengthening of the cell cycle induced by 2,4-D.

1.4.2.2.2 Disorganised growth and genetic change

Tissue culture removes cells from their normal environment, and in the more 'disorganised' culture systems such as callus culture, it has been suggested that there is a reduction in the strictness of control over the sequence of DNA synthesis, nuclear division and cytoplasmic division. This could produce chromosomes with more chromatids than usual, nuclei with altered chromosome number, and multinucleate cells (Partanen, 1963). Hormones may indirectly induce mitotic abnormalities as a result of their effects on cell growth and division (Bayliss, 1980).

If such genetic abnormalities are the result of disorganised growth *per se* , then similar variation might be expected to occur in other systems involving disorganised growth. Indeed, chromosomal variability has been reported in the propagation of *Pelargonium* via cuttings that involve callus formation (Skirvin and Janick, 1976) and in crown gall tumours (Mouras *et al.*, 1987).

1.4.2.2.3 Stress

Extreme environmental conditions, such as high temperature, water-logging, lack of oxygen and high salinity can lead to changes in gene expression (Sachs and Ho, 1986), and the changes in gene expression observed in cultured cells (for example, changes in anthocyanin production (Dougall *et al.*, 1980) and in growth regulator requirements (Meins and Binns, 1977)) could be a response to the alterations that occur in environmental conditions in culture. Environmental conditions can have a more permanent effect too: for example, heritable changes in the copy number of repeated sequences, including rDNA, occurred in two flax lines grown in different nutrient conditions (Cullis and Cleary, 1986b) in cultured flax cells (Cullis and Cleary, 1986a) and following crosses between two flax genotrophs (Durrant, 1981). Several other examples of genetic instability following hybridisation are cited by Walbot and Cullis (1985).

The high incidence of mutations in wide crosses is superficially similar to hybrid dysgenesis, that is, the deterioration of hybrid lines, in *Drosophila*. The many genetic abnormalities associated with this phenomenon, including chromosomal rearrangement and non-disjunction, are thought to be caused by the activation of cryptic transposable elements (Bregliano and Kidwell, 1983). McClintock (1984) has suggested that transposition is also responsible for the instability observed in wide crosses. Other types of "genomic shock" may elicit similar responses: transposition has been reported following viral infection (Mottinger *et al.*, 1984) and after irradiation (Dellaporta and Chomet, 1985). The similarity between the chromosomal abnormalities seen in these cases and those found in tissue culture-derived plants suggests that transposition may be a common response to stress and that it plays an important role in somaclonal variation. Further evidence to support this hypothesis is given in Section 1.3.6.

In summary, the initial explant may contain a mixture of cell genotypes, but variation can also arise *in vitro* , either from abnormal growth patterns or from stress.

1.5 FACTORS AFFECTING SOMACLONAL VARIATION

1.5.1 The plant

1.5.1.1 Species

Some plant species are more variable *in vivo* than others, and the extent of somaclonal variation may reflect this inherent variability. For example, maize, which has many restriction sites that vary between inbred lines (Helentjaris *et al.*, 1985) and many transposons (Döring and Starlinger, 1986), also seems to have a high level of somaclonal variation. Recently formed hybrids may be particularly prone to chromosomal rearrangement *in vivo* (McClintock, 1984), which may explain their instability *in vitro*. For example, 4 out of 10 plants regenerated from a wheat × rye culture had translocations (Lapitan *et al*, 1984). Plants which do not show polysomaty *in vivo* also tend to be more cytogenetically stable *in vitro* (D'Amato, 1985).

1.5.1.2 Ploidy

The ploidy of a plant species seems to influence both the frequency and the types of chromosomal aberrations occurring *in vitro*. 'Diploidisation' of cultures from haploid *Crepis capillaris* plants occurred more rapidly and more frequently than 'tetraploidisation' of cultures from diploid plants (Sacristán, 1971), suggesting that the haploid state is inherently less stable than the diploid state. Karp *et al.* (1984) found that all monohaploid potato plants, but only 50-60% of dihaploids, underwent chromosomal doubling in culture. More aneuploidy occurred in regenerants from cultures of tetraploid *Solanum tuberosum* (Karp *et al*, 1982) than in those from cultures of dihaploid plants

(Wenzel *et al.*, 1979). In general, polyploid plant species seem to be more prone to changes in chromosome number than diploid species. This difference may be due to the buffering capacity conferred by the extra copies of the genome in polyploids, which could impart a greater tolerance of change.

1.5.1.3 Genotype

Different genotypes within a species can have different degrees of stability *in vitro*. McCoy *et al.* (1982) compared two varieties of oats and found that 48% of the plants regenerated from 4 month-old 'Lodi' tissue cultures had cytologically detectable abnormalities, compared to only 12% of those from 'Tippencoe' cultures of the same age. Karp *et al.* (1982) found that 30% of regenerants from cultures of the potato cultivar 'Fortyfold' retained the normal number of chromosomes compared to less than 4% of 'Maris Bard' regenerants. Also, the range of aneuploid chromosome numbers found was much greater in 'Maris Bard'.

1.5.2 The culture system

1.5.2.1 The explant

The heterogeneity of a culture will depend partly on the heterogeneity within the explant: if cells containing somatic mutations (section 4.1.1) or abnormal chromosome numbers (section 4.1.3) respond to culture, the initial culture may be composed of a mixture of genetic types of cells. The origin of the explant may thus influence the nature of cells in the culture. For example, Kasperbauer and Collins (1972) studied plants regenerated from cultures initiated from different tissues of haploid tobacco plants. Midveins from young leaves only gave rise to haploid plants, whereas midveins from older leaves produced a mixture of haploid and diploid plants. Nearly all plants regenerated from stem pith were aneuploid, either at the diploid or the tetraploid level.

1.5.2.2 The culture regime

Some types of tissue culture seem to produce lower frequencies of somaclonal variation than others. For example, meristem culture is often reported to produce highly uniform plants (Hu and Wang, 1983). The apparent stability may be related to the high degree of multicellular organisation retained in such cultures, rather than to an intrinsic stability of meristematic cells: excised pea meristems, when macerated and placed onto callus induction medium, soon gave rise to haploid, aneuploid and polyploid cells (Natali and Cavallini, 1987). The genetic stability of meristems *in vitro* may be due to the strict control of the sequence of DNA replication and cell division retained in the intact tissue. It has been proposed that there exists in meristems a mechanism to eliminate mutant cells (''diplontic selection''— Gaul (1961))—; this would explain phenomena such as the production of diploid spikes on aneusomatic *Durum* wheat plants *in vivo* (Lupi *et al.*, (1981)). It is possible that such a mechanism could explain the stability of meristems *in vitro*. Klekowski (1988), though, believes that the fate of a mutant cell will depend on its fitness compared with non-mutant cells, and on where in the meristem the mutation occurs; a mutation that had a neutral or beneficial effect on cell growth and division would be propagated if it occurred in a region of the meristem that divided further. The degree of dedifferentiation that occurs *in vitro* thus seems to be an important factor in genetic stability.

1.5.2.3 Selection and evolution in culture

The proportion of mutant cells in a culture will depend both on the rate of production of new mutants and on the ability of the mutant cell types to survive and proliferate *in vitro*. The spectrum of variation in the regenerated plants will also depend on the capacity of the mutant cell types for organogenesis.

The tissue culture environment can be seen as a whole array of selective forces, both physical and chemical, that will influence the make-up of the cells in the culture. Deliberate selection for a particular mutant type has often been used to isolate particular biochemical mutants, but other factors, such as hormones and subculture intervals, can also determine which cell types come to dominate (Bayliss, 1980). Where dedifferentiation is complete, the cells are freed from the constraints of life within the plant, and the 'fittest' cells — those that can multiply fastest in the given conditions — will tend to predominate.

A further analogy to the evolutionary process can be made: each subculture will select a small sample of the cells from the original culture; if these cells are not 'representative' of the original culture then **genetic drift** will occur. Such evolution of cell types was found in tobacco callus culture by Floh and Handro (1985).

1.6 GENETIC VARIATION IN ANIMAL CELL CULTURE

Genetic instability is not confined to plant cell and tissue culture; it is also a well-known phenomenon in animal cell cultures. Extensive structural and numerical chromosome variation occurs in cultures of malignant and non-malignant human cells (reviewed by Hsu (1961)). Some types of culture are reported to be predominantly diploid until they enter the final, ageing, phase when the frequency of tetraploidy and chromosomal aberrations increases (Saksela and Moorhead, 1963). Spontaneous drug-resistant mutants have also been isolated from animal cell cultures (Littlefield, 1976); a more recent example—that of methotrexate resistance in a mouse cell culture has been discussed in Section 1.3.5.

As in plant cell cultures, some of the variation is thought to derive from variant cells *in vivo*. Complex evolutionary changes also occur in the cell population *in vitro*, with a tendency to polyploidisation that leads Hsu (1961) to suggest that cells with extra chromosomes are more tolerant of chromosomal abnormalities than diploid cells, and therefore survive and proliferate better in the environment *in vitro*.

The aims of this project are to monitor the extent of somaclonal variation in tobacco plants derived from callus culture, and to investigate the nature and origin(s) of this variation. It is intended that techniques for RFLP analyses will be developed and applied to the quantification of genetic variation between plants representing different levels of expected divergence, with the ultimate aim of developing a system for germplasm identification, particularly with respect to tissue culture-derived plant lines. For comparison of the relative values of different techniques in the analysis of somaclonal variation, morphological and cytogenetic variation will also be investigated.

1. REGENERATION OF N. TABACUM FROM TISSUE CULTURE

This section outlines the protocols used to regenerate *Nicotiana tabacum* plants from tissue culture, and describes callus induction and regeneration.

1.1 Materials and methods

1.1.1 Plant material

1.1.1.1 Sources

Seeds from *Nicotiana tabacum* cultivars 'Xanthi' and 'White Burley', and from other *Nicotiana* species, were obtained from the University of Bath seed collection. Other seed samples were kindly given by Dr. J.Draper, University of Leicester (SR1), J.Nicol, John Innes Institute, Norwich ('Samsun') and T.Mitchell, B.A.T. Ltd., Southampton ('Virginia' and 'Western').

1.1.1.2 Growth conditions

Seed was sown on 'Levington's Universal' compost and was germinated at 25°C in a mist-house. Seedlings were transplanted into 'Levington's Universal' compost in 3.5'' pots, and grown in a greenhouse at 25°C ±4°C in a 16 hour light / 8 hour dark photoperiod.

1.1.1.3 Self-fertilisation and seed collection

In order to prevent cross-fertilisation, each flower was enclosed in a double layer of fine muslin before the flower opened, allowing room for the full expansion of the flower. Seed capsules were left on the plants until fully dry before seed collection.

1.1.2 Tissue culture protocols

1.1.2.1 Explants

Young, fully-expanded leaves from pre-flowering, greenhouse-grown 'Xanthi' plants were washed carefully with water, surface-sterilised in 15% (w/v) sodium hypochlorite for 20 minutes and then rinsed 4 times in sterile distilled water. In a laminar flow cabinet, 1cm^2 leaf pieces were cut from the leaves, avoiding major veins; the leaf squares were then transferred to the appropriate medium (see Section 2.1.2.2). Hypocotyl cultures were established by germinating surface-sterilised seeds on moistened, sterile filter paper, then excising a 1cm long portion of the hypocotyl from the germinated seedlings one week later. Culture media were as for the leaf explants (see below).

1.1.2.2 Tissue culture media

Powdered Murashige and Skoog medium (MS; recipe given in Appendix 1) was obtained from Flow Laboratories Ltd. (Irvine, Scotland) and reconstituted according to the manufacturer's instructions. Sucrose, plant growth regulators and casein hydrolysate (where appropriate) were added (see below), the pH was adjusted to 5.6, and 0.8% (w/v) agar (Oxoid,Technical grade) was added. The medium was then sterilised by autoclaving at 15lb/in^2 (121°C) for 15 minutes, and dispensed into 5cm diameter, deep Petri dishes (for tissue culture) or 100ml screw-top jars (for rooting shoots).

Composition of tissue culture media: basic MS medium was prepared as described above, and various additives were included to make up the different media:

i. callus induction medium (CIM) : 2% (w/v) sucrose, 2g/l casein hydrolysate and 4.5µM 2,4-D

ii. callus maintenance medium (CMM) : 2% (w/v) sucrose, 4.5µM 2,4-D

iii. regeneration medium (RM) : 2% (w/v) sucrose, 5µM BAP

1.1.2.3 One-step regeneration

Leaf explants were placed directly onto regeneration medium, and subcultured onto fresh medium every 4 weeks.

1.1.2.4 Two-step regeneration

Leaf explants were placed on CIM for 4 weeks, then transferred to CMM for further callus growth. They were subcultured every 4 weeks onto fresh CMM until regeneration was desired; then, the callus was transferred to regeneration medium.

1.1.2.5 Rooting and transfer to greenhouse

Shoots were transferred to half-strength MS medium, with 1% (w/v) sucrose, for rooting. Rooted shoots were transplanted into 3.5'' pots of 'Levington's Universal' compost and then grown as for seed-grown plants (see Section 2.1.1.2).

1.1.2.6 Culture conditions

All cultures were kept at 25°C ±2°C with a 16 hour light / 8 hour dark photoperiod, and a light intensity of 3000 lux.

1.2 Experimental work, results and discussion

Plants were regenerated from tissue cultures according to the protocols described in Section 2.1.2. The culture regimes used are summarised in Figure 3.

1.2.1 Responses of explants to different culture regimes

1.2.1.1 One-step regeneration

The explant increased in size, swelling in all dimensions, indicating that either cell division or cell expansion (e.g. by water intake) had occurred. Most of each explant seemed to retain its organisation, and callus formation was restricted to the cut edge of the explant. Compact, white callus was formed at the cut edges of veins; one end of the vein always produced more callus than the opposite end — this is probably a consequence of an auxin gradient within the vein. Brossard (1975) noted that in tobacco pith, only the basal ends of explants tended to produce callus *in vitro*; she suggested that this was due to the establishment of IAA gradients in explants after excision, because of a natural tendency for basipetal auxin transport. Faster growing, green callus developed especially at the corners of the explant. This callus did not apparently arise from vascular tissue, and produced many shoots (Plate 1). I suggest that the latter type of callus arose from leaf mesophyll tissue whereas the former came from veins; histological examination would be necessary to confirm this.

Callus formation and shoot regeneration can evidently occur without the addition of exogenous auxin. This one-step regeneration was rapid and the response was vigorous. Regeneration did not appear to be direct, as can occur in some species (for example, *Begonia* species (Broertjes *et al.*, 1968) but *via* a callus phase.

1.2.1.2 Two-step regeneration

1.2.1.2.1 Intact leaf explants

In contrast with the explants placed directly onto regeneration medium, those placed onto callus induction medium did not swell up. Compact callus formed at cut vein edges (Plate 2). After subculture to callus maintenance medium, almost liquid, spreading pale brown callus formed around the edges of the explants, with patches of other callus types— a more compact, rough-surfaced type and a smooth, globular, white callus (Plate 3A). Different explants varied in the proportions of these

Figure 3: Summary of tissue culture regimes used

CIM= callus induction medium, CMM= callus maintenance medium,
RM= regeneration medium

One-step regeneration

LEAF EXPLANT → RM

Two-step regeneration

LEAF EXPLANT → 4 WEEKS ON CIM → 4 WEEKS ON CMM → RM "INTACT"

LEAF EXPLANT → REMOVE LOWER EPIDERMIS → 4 WEEKS ON CIM → 4 WEEKS ON CMM → RM "PEELED"

SHORT TERM

LEAF EXPLANT → 4 WEEKS ON CIM → 3×4 WEEKS ON CMM → RM

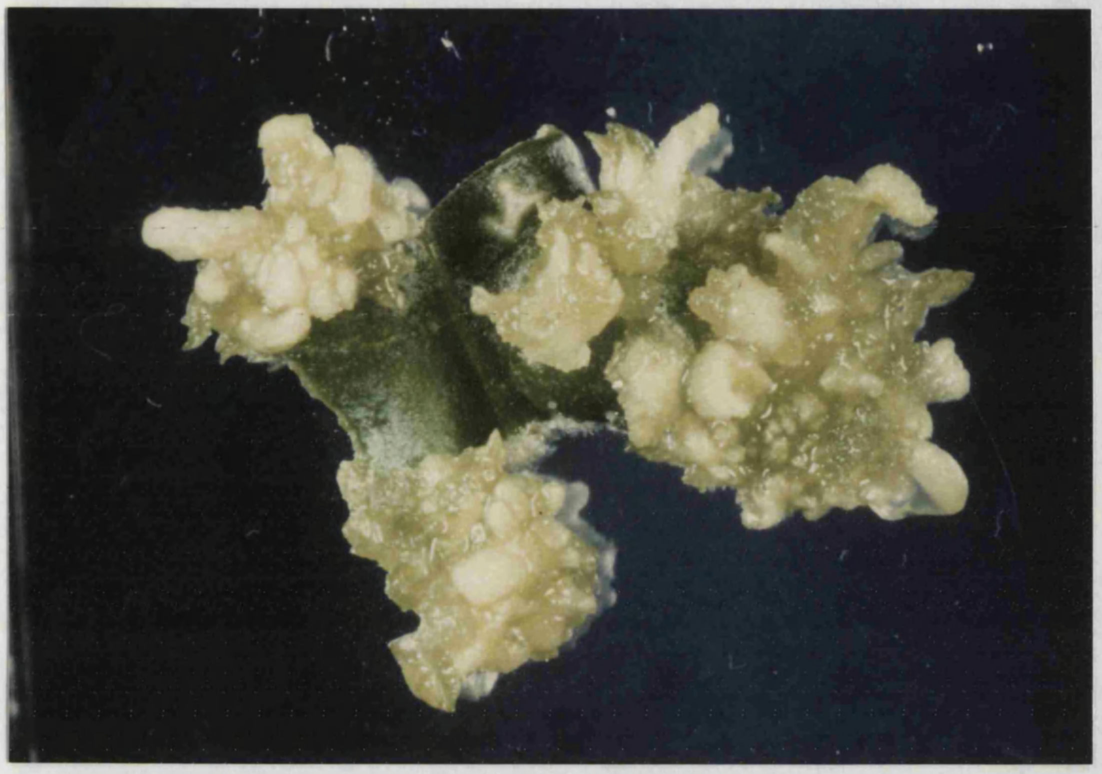
HYPOCOTYL EXPLANT → 4 WEEKS ON CIM → 3×4 WEEKS ON CMM → RM

LONG TERM

facing page 26

Plate 1:

One-step regeneration of shoots from leaf explants of *Nicotiana tabacum*, after 21 days on regeneration medium (× 4.5)



different types of callus. The regeneration response was less than in the one-step system. It is illustrated in Plate 4A.

1.2.1.2.2 Peeled leaf explants

Explants that had had their lower epidermis removed swelled in all dimensions, mostly in thickness, and the exposed surface became shiny and uneven, with some browning. Callus formation occurred at the cut end of veins. After 3 weeks on callus maintenance medium, the original explant was much less distinct than in the intact explant system (Plate 3B). Callus had developed over the whole "peeled" surface. The morphology of the callus varied from rough globules of pale callus to less structured, dark brown callus.

1.2.1.2.3 Long-term leaf cultures

After several subcultures, callus morphology was very variable between cultures and even within a single culture (Plate 4B). Such variability has also been observed by Floh and Handro (1985) and is thought to be due to a process analogous to genetic drift that occurs when a heterogeneous population of cells is subcultured.

Note: In all cultures, some of the regenerated shoots seemed to dominate, growing rapidly whilst many other shoots remained tiny (Plate 4A). In order to avoid bias in the selection of shoots, large shoots were periodically removed for rooting, and then the explant or callus was replaced on regeneration medium to allow further development of the small shoots.

facing page 28

Plate 2:

Callus formation at cut edge of vein on leaf explant after 7 days on callus induction medium (× 7.4)

Plate 3:

Callus formation after 4 weeks on callus induction medium and 1 week on callus maintenance medium
(A): Intact explant ($\times$ 2.1)
(B): Explant with lower epidermis removed prior to culture ($\times$ 2.1)

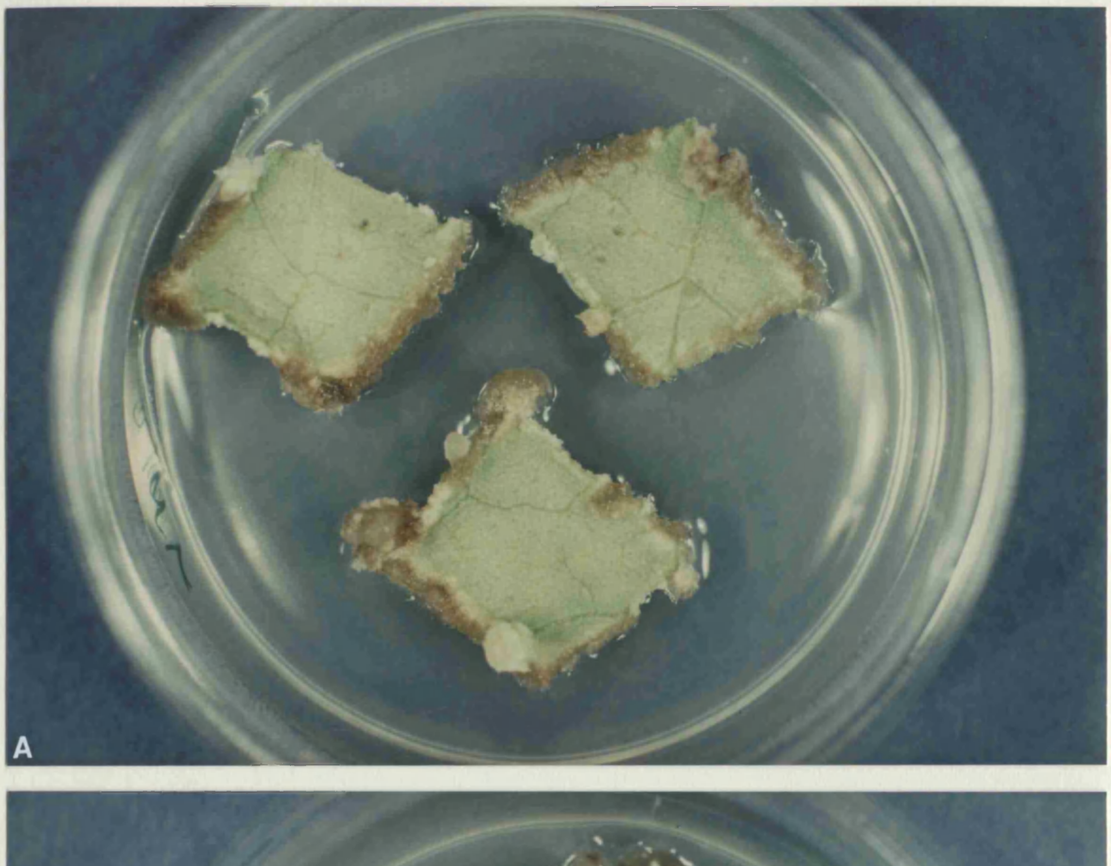
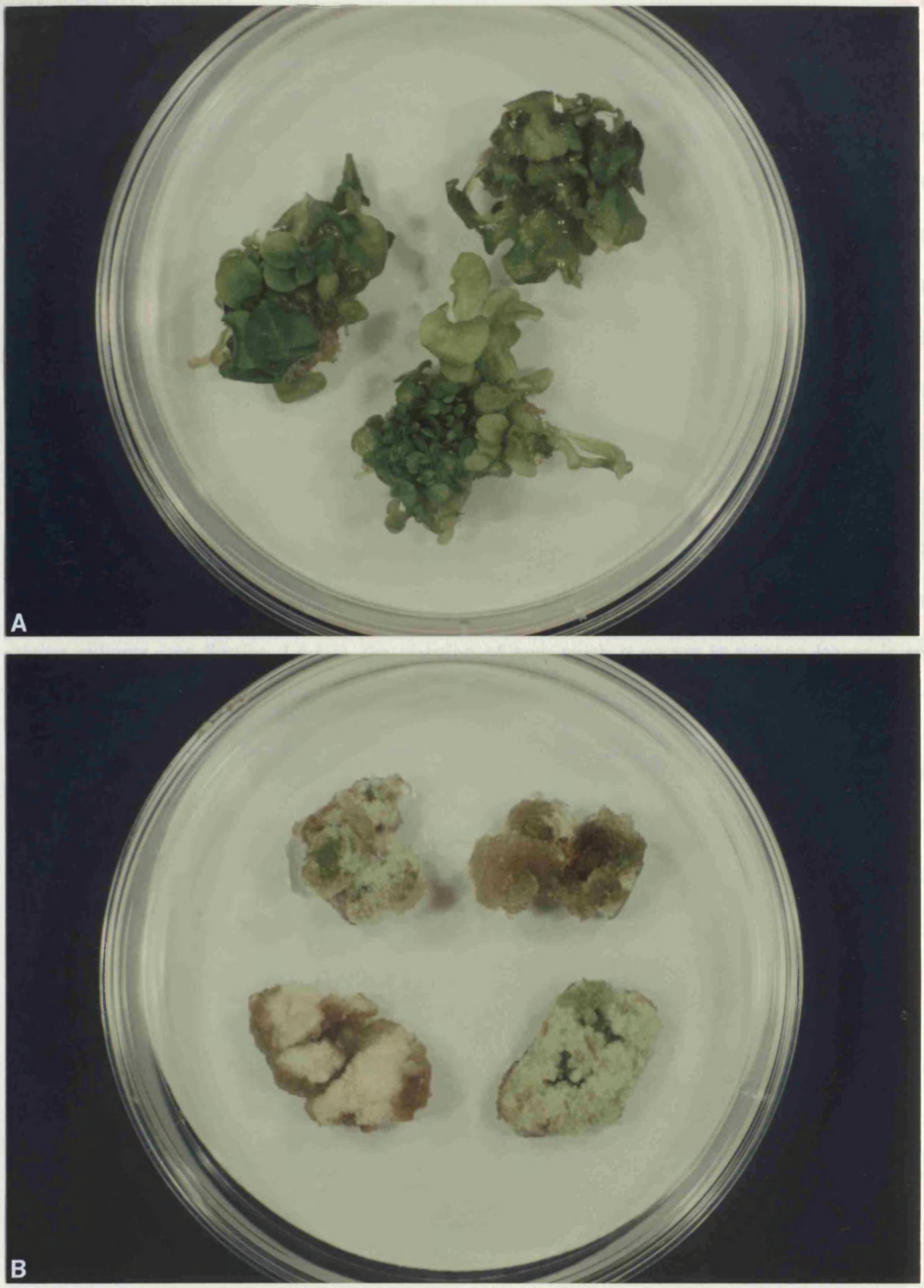

A
B

Plate 4:

(A): Regeneration of shoots *via* callus after 3 weeks on regeneration medium ($\times$ 2.1)
(B): Morphology of callus from leaf explants after long-term culture on callus maintenance medium ($\times$ 2.1)


A
B

1. MORPHOLOGICAL AND CYTOGENETIC ANALYSES OF REGENERATED PLANTS

1.1 INTRODUCTION

Tobacco has been widely used for tissue culture studies because of the ease with which it regenerates, and consequently there are many reports of its behaviour *in vitro*. Chromosomal abnormalities, including polyploidy, aneuploidy and mixoploidy, have been reported in regenerated plants (Murashige and Nakano, 1967; Nuti Ronchi *et al.*, 1981). Heritable variation in quantitative traits, for example, height, have also been described (Prat, 1983). Many abnormalities have been observed in cultured cells, such as multiple nuclei (Nuti Ronchi *et al.*, 1973), fissured and fragmented nuclei (Brossard, 1975) and numerical chromosome changes (reviewed by Brossard, 1975). Most of these reports have concerned callus cultures derived from stem pith, which is known to contain polyploid cells *in vivo* (Murashige and Nakano, 1967 and Brossard, 1975). The genetic status of tobacco *leaves*, both *in vivo* and *in vitro* is less well established. As endopolyploidy can be restricted to specific tissues (D'Amato, 1977), and as it may be an important determinant of the degree of polyploidy in tissue cultures (see Section 1.4.1.3), the question of how much it occurs in tobacco leaves is an important one if we are to have a complete understanding of somaclonal variation in tobacco.

This section of the project aimed to assess the extent of variation in morphology and in chromosome number in tobacco plants regenerated from leaf explants, and to establish how much of the morphological variation had a genetic basis. To gain an insight into the origin of somaclonal variation, different culture systems were compared and the genetic uniformity of the original explant was investigated.

1.2 MATERIALS AND METHODS

1.2.1 Cytogenetic analysis of regenerated plants

Root tips of young, healthy plants were washed to remove compost, then placed in 3mM 8-hydroxyquinoline at 18°C for 4.5 hours. The root tips were then rinsed in distilled water and transferred to freshly made fixative (3 parts 95% ethanol to 1 part glacial acetic acid) and kept at 4°C for at least 24 hours. After hydrolysis for 5 hours at room temperature in 1N HCl, the roots were washed with 0.1M sodium acetate (pH 4.5) and then placed in carbol fuchsin solution D (see Appendix 2) overnight.

The meristematic region was excised, then macerated in a drop of carbol fuchsin on a microcope slide and squashed beneath a cover slip. Cells were examined using an Olympus BH-2 microscope.

1.2.2 DNA content counting

1.2.2.1 Protoplast isolation

This was based on the method of Power and Cocking (1970). Fully expanded *Nicotiana tabacum* 'Xanthi' leaves were surface-sterilised by immersion in 10% (v/v) sodium hypochlorite solution for 20 minutes, then rinsed 4 times in sterile distilled water. The midrib was excised and sliced in two longitudinally, then placed in a Petri dish of CPW salts (see Appendix 1) with 11% (w/v) mannitol for one hour to plasmolyse. The lower epidermis was peeled off from the remainder of the leaf, then any major veins were removed and the leaf pieces were floated, exposed leaf mesophyll downwards, on CPW salts with 11% (w/v) mannitol for one hour. The CPW solution was then removed with a hypodermic syringe, and replaced with a filter-sterilised solution of 4% (w/v) "Onozuka R-10" cellulase and 0.4% (w/v) "Macerozyme"*. The leaf pieces were incubated in the enzyme solution in the dark at 25°C overnight. Each Petri dish was then swirled gently to release the protoplasts, then the debris was removed and the protoplasts were carefully transferred to sterile centrifuge tubes using a Pasteur pipette. The protoplasts were pelletted by spinning very gently (c. 200 rpm) in a Fisons 'Chilspin' centrifuge for 5 minutes, then resuspended in CPW salts with 20% (w/v) sucrose. The

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* The enzymes were obtained from Yakult Honsha Co. Ltd., Tokyo, Japan.

centrifugation step was then repeated: intact protoplasts float on 20% sucrose and can thus be separated from debris and broken cells. The layer of intact protoplasts was transferred to a fresh tube, diluted with CPW + 11% mannitol, then spun down again and resuspended in 5ml CPW + 11% mannitol.

3.2.2.2 Fixation

To one volume of the protoplast suspension (Section 3.2.2.1) was added four volumes of fixative (adapted from Magnien *et al.*, 1982: CPW salts without KNO_3, 300mM sorbitol and 10% (w/v) formaldehyde).

3.2.2.3 Staining

1ml of fixed protoplast suspension was filtered through a 5µm membrane (Millipore) then allowed to air-dry completely. The membrane was then immersed in a series of baths at room temperature:

1	5N HCl (55 minutes)
2	H_2O rinse
3	Feulgen reagent (see Appendix 2) (1.5 hours)
4	SO_2 water (see below) (5 minutes each)
6	H_2O (5 minutes each)
8-18	5 minutes each in a series of ethanol baths (see Table 1)
19	xylol (5 minutes)

Finally, the membrane was mounted in Canada balsam.

SO_2 water: 5ml 1N HCl, 5ml 10% (w/v) $K_2S_2O_5$, 100ml H_2O

3.2.2.4 Densitometry

DNA content was estimated by measurement of the absorbance at λ = 530-560nm using a Leitz MPV 3 microdensitometer. For each cell, the absorbance of the nucleus was measured, followed by that of a cytoplasmic region, keeping the measuring aperture constant throughout.

1.3 EXPERIMENTAL WORK, RESULTS AND DISCUSSION

1.3.1 Cytogenetic analysis

The chromosome numbers of 24 plants regenerated from various culture regimes and 10 seed-grown 'Xanthi' plants were determined by examination of mitotic metaphases on root tip cells (Section 3.2.1) and the morphology of each plant was noted. The results are shown in Table 2, and examples of chromosome spreads and plant morphology are shown in Plates 5 to 7.

It can be seen that high chromosome number is associated with characteristic phenotypic abnormalities. Similar results have been reported in tobacco by Murashige and Nakano (1966) and by Syono and Furuya (1972); the former authors described polyploid plants as smaller than normal, with darker green, rounder leaves, thicker stems and larger flowers. The latter noted imperfect fusion of petals into the corolla tube in plants regenerated from callus culture, but did not analyse the chromosome constitution of these plants. The correlation of polyploid chromosome numbers with such phenotypic variation means that it is valid to use morphological analysis to estimate the extent of polyploidy. Aneuploids at the diploid level, however, are harder to recognise: although their fertility is often reduced, they may be otherwise very similar to normal diploid plants. Detailed descriptions of the appearances of the monosomic types of *Nicotiana tabacum* are available (e.g. Smith, 1979), but it would require extensive analysis to make these the basis of a morphological test for aneuploidy; such analysis is beyond the scope of this study. It must also be noted that reduction in fertility is not a completely reliable indicator of numerical chromosome variation, as it was observed in one diploid plant (see Table 2). This may have been due to a structural chromosome abnormality which would not have been detected.

In conclusion, then, morphological analysis could be useful for large-scale screening for high chromosome number, but would be harder to apply to the detection of aneuploidy at the diploid level.

The results of these preliminary investigations confirm the findings of other authors that there are polyploid and aneuploid plants among those regenerated from tissue culture. In this small sample, aneuploidy was common; such a frequency of plants with 'unbalanced' genomes seems to be typical of polyploid plants (for example, aneuploidy is common in protoplast-derived tetraploid potato (Karp *et al.*, 1982)) and so may be a reflection of the amphidiploid nature of *Nicotiana tabacum*. This

TABLE 1. Composition of wash solutions 8-18

WASH	% (v/v)	
	BUTANOL	ETHANOL
8	0	5
9	0	10
10	5	15
11	10	20
12	15	25
13	25	30
14	40	30
15	55	25
16	70	20
17	85	15
18	100	0

TABLE 2. Chromosome numbers and associated morphological variation

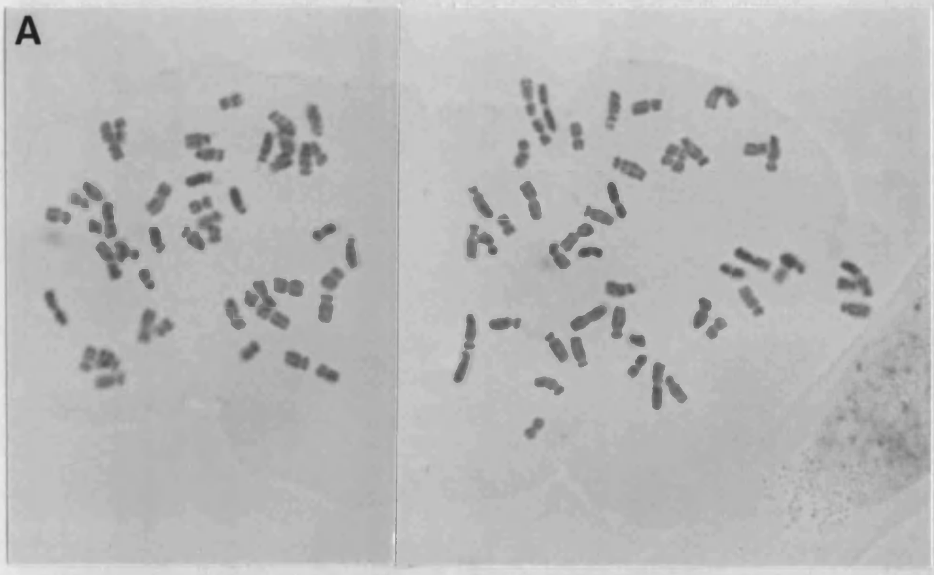
The chromosome numbers of 24 regenerated plants and 10 seed-grown control plants were determined by carbol fuchsin staining of root-tip mitoses. The morphology of plants is compared to their chromosome constitution.

PLANT SOURCE	CHROMOSOME CONSTITUTION		APPEARANCE OF PLANT	FREQUENCY	TOTAL
	CATEGORY	NUMBER OF CHROMOSOMES			
SEED	DIPLOID	2n=48	Normal	10	10
TISSUE CULTURE	DIPLOID	2n=48	Normal	10	11
			Small flowers with reduced fertility	1	
	2n<48	(i) 42	Normal	1	3
		(ii) 44	Normal	1	
		(iii) 35	Narrow leaves and seed pods, tiny flowers, low seed yield	1	
	2n>48	(i) 51	Small flowers, very low pollen yield	1	7
		(ii) 55	Unfused flowers, low pollen yield	1	
		(iii) >60	Unfused flowers with some anthers fused to petals, low pollen and seed yields. Seed large and very dark brown	1	
		(iv) 65*	Large flowers with wide corolla tube; low yield of large rough-surfaced seed	1	
		(v) 68	Short. Coarse leaves with prominent veins. Large, incompletely fused flowers with protruding anthers. Stigma low in corolla tube and often 3-lobed. Low pollen yield.	1	
		(vi) 80	Large flowers with wide corolla tube. Reduced pollen yield; pollen looked wet and clumped together. Large, rough surfaced seed.	1	
		(vii) 96	Thick, coarse, roundish leaves with prominent veins and paler coloured sectors. Large flowers.	1	
	MIXOPLOID	(i) 45,48	Normal	1	3
		(ii) 42,43,50	Normal	1	
		(iii) 48,64	Small flowers with very low pollen and seed yields	1	

* chromosomes appeared to be clumped together, and some nuclei were lobed

Plate 5:

(A): Mitotic metaphase in two normal, diploid cells (2n=48) from the root tip of a regenerated *Nicotiana tabacum* plant (× 3000)
(B): Karyotype of the cell above right, showing the 24 pairs of chromosomes; one pair (SAT) is satellited. Diagrammatic interpretations of overlapping chromosomes are shown. (× 6000)


A

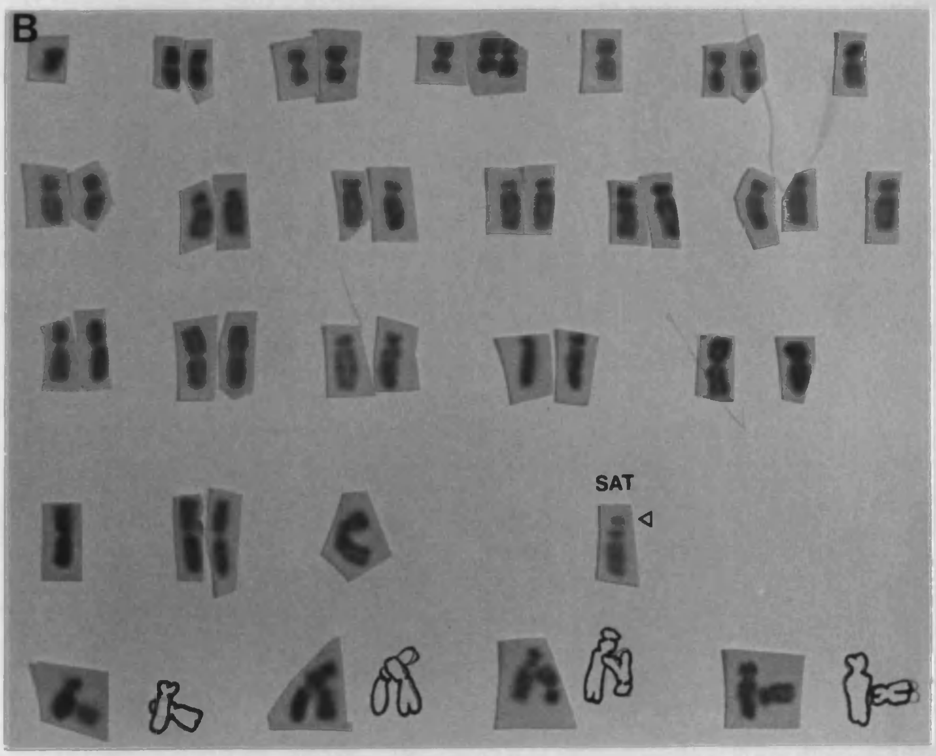

B
SAT

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Plate 6:

Polyploid cell with approximately 76 chromosomes from the root tip of a regenerated *Nicotiana tabacum* plant (× 3000)

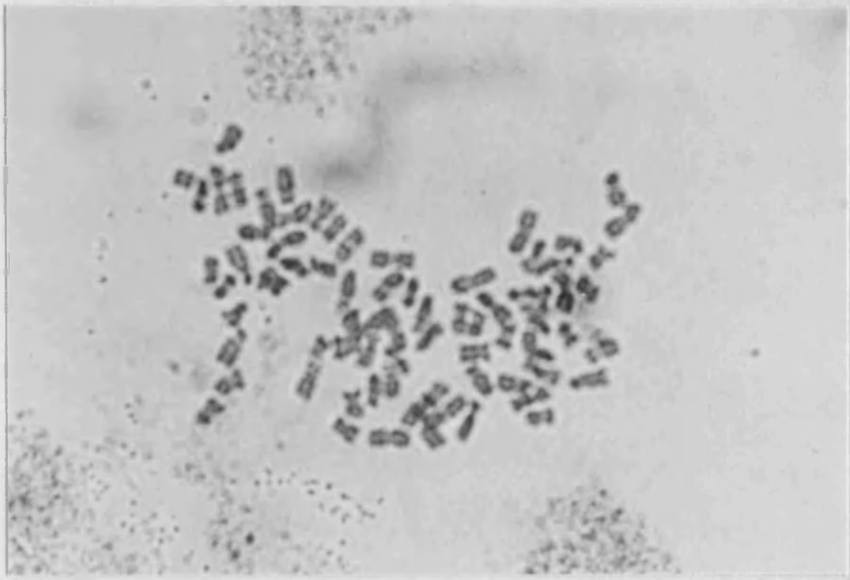

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Plate 7: Flower morphology in tobacco plants with normal and high numbers of chromosomes

(A): Extreme incomplete fusion of petals in aneuploid plant at the tetraploid level (2n=68) (× 1.7)

(B): Large, sub-fertile polyploid flower (right, 2n=96) with control (2n=48) (× 2.3)


A
B

ability of *N. tabacum* to tolerate variable chromosome number *in vitro* may be compared to the diversification of chromosome number observed in the genus *in vivo*. Although many *Nicotiana* species have either 12 or 24 pairs of chromosomes, some have other numbers thought to arise from chromosome loss following hybridisation (Goodspeed, 1954). Mixoploidy has also been reported previously in tobacco (Nuti Ronchi *et al.*, 1981). It could be due to the formation of a shoot from more than one cell in the culture, but, especially where *several* different chromosome numbers are found within one plant, I think that it is more likely to arise due to non-disjunction of chromosomes during shoot development. The extent of chromosomal variation in regenerated plants may be an underestimate of the amount of variation arising in culture: shoot regeneration, and the rooting and maturation of these shoots, are further "screens" for normality. For example, Nuti Ronchi *et al.* (1981) compared the chromosome constitutions of the shoots produced on *Nicotiana glauca* tissue cultures with those of shoots that successfully rooted and grew to maturity. They found that 20.7% of the shoots were aneuploid or mixoploid, compared to only 1.6% of the regenerated plants, indicating that an unbalanced genome hinders plant growth and development.

No further work was done on aneuploidy at the diploid level or on mixoploidy, because of the difficulty in estimating the frequencies of these conditions without cytogenetic analysis.

1.3.2 Morphological analysis

1.3.2.1 Qualitative traits

1.3.2.1.1 Assessment of qualitative traits

A total of 424 regenerated plants and 111 control seed-grown plants were screened for variation in a number of qualitative traits. The results are summarised in Table 3.

It can be seen that while there was a high level of uniformity in control seed-grown plants, variation for a number of traits occurred in regenerated plants. More detailed analyses give a clearer picture of the nature of these changes:

(i) In a preliminary investigation (Section 3.3.1), some characteristic morphological features were found in plants with abnormally high chromosome number. Several of the changes noted in Table 3 are the same as those found in the earlier experiment, for example, incomplete fusion of petals into the

TABLE 3. Assessment of qualitative trait variation in regenerated plants

The number of variant plants obtained in different phenotypic categories is shown for each culture system and for control, seed-grown plants.

PHENOTYPE	CONTROL	REGENERATION SYSTEM					TOTAL excluding control
		ONE-STEP	TWO-STEP				
			SHORT-TERM		LONG-TERM		
			INTACT	PEELED	LEAF	HYPOCOTYL	
Yellow-green leaves	0	2	0	1	0	0	3
Variegated leaves	0	0	2	1	1	0	4
Coloured sectors on leaves	0	4	0	3	0	2	9
Pointed leaves	0	0	2	1	5	3	11
Coarse leaves with prominent veins	0	12	1	10	4	9	36
Cupped leaves	0	0	0	1	3	0	4
Strong constriction at base of leaf	0	0	2	1	0	0	3
Thin stem	1	2	2	2	2	0	8
Thick stem	0	4	3	2	0	1	3
Long 'wings' from leaf run down stem	0	1	0	1	1	0	3
Hairy	0	7	1	0	2	0	10
Fasciated	0	52	3	5	2	2	54
Small flowers	1	4	1	2	1	4	12
Large flowers	0	3	6	12	0	2	23
Incompletely fused flowers	0	1	0	4	1	7	13
Dark pink flowers	0	0	1	0	1	3	5
Narrow seed pods	0	1	0	2	2	6	11
Pale seed pods	0	0	0	0	1	2	3
Poorly filled seed capsules	0	5	1	2	0	8	16
TOTAL NO. OF ABNORMALITIES a	2	98	25	50	25	49	238
TOTAL NO. OF ABNORMALITIES b	2	46	22	45	23	47	184
Number of normal plants	110	157	35	43	28	3	376
TOTAL NUMBER OF PLANTS	111	212	57	83	45	27	424
Mean number of abnormalities per plant a	0.018	0.462	0.438	0.602	0.555	1.815	0.561
Mean number of abnormalities per plant b	0.018	0.217	0.385	0.542	0.511	1.741	0.434

Key: a including fasciation, b excluding fasciation corolla tube, and coarse leaves with prominent veins. This indicates that some of the morphological variation observed in the later study was due to chromosomal variation.

(ii) If those changes associated with high chromosome number are ignored, there is still a significantly higher level of variation in regenerated plants compared to the controls (Table 4). To investigate whether such variation had a genetic basis, inheritance tests were carried out. 100 progeny of each variant, obtained by self-fertilisation (Section 2.1.1.3), were screened for the variant traits. The results are shown in Table 5.

Ten out of the eleven traits tested were not inherited. The observed changes must, therefore, have been due to physiological or biochemical alterations, which may have been brought about by changes in gene expression. It is possible that the abnormal levels of plant hormones in the tissue culture media persist in the plants and thus alter the developmental signals present. From Table 4, it can be seen that the frequency of qualitative variation differed significantly between culture regimes. This could have been due to the varying conditions and lengths of time in culture; for example, the longer term cultures would have been exposed to hormones for longer and so may have developed more abnormal internal hormone levels.

Lack of inheritance of a trait is not absolute proof that the trait has a non-genetic basis: in the case of chimaeric plants, mutant and normal cells could 'sort out' during development, so that only normal cells took part in floral induction. Such a phenomenon has been demonstrated in chromosomally mosaic *Durum* wheat plants (Lupi *et al.*, 1981). Chimaerism can only be easily detected if it has an obvious effect on the phenotype: most reports concern leaf variegation (e.g. Burk *et al.*, 1964). Other chimaeras may not be very different from normal plants; for example, differences in growth rate between the different layers of cells in a leaf can produce leaf crumpling and rolling up of leaf edges (Tilney-Bassett, 1986). The "cupped leaf" phenotype, illustrated in Plate 8, could be such a chimaera. The only trait that was inherited — the small flower phenotype — behaved like a homozygous mutation. Further analysis would be necessary to elucidate the precise nature of the changes that occurred, including analysis of large numbers of the progeny of the variant plants, and cytogenetic analysis. Screening the progeny of all regenerated plants would also be necessary to detect recessive mutations in the heterozygous state.

TABLE 4. Qualitative variation in plants with 'normal' chromosome number

This table compares the frequency of phenotypic variation in plants regenerated from different culture systems; fasciation and those alterations previously found to be associated with high chromosome number are not included.

				TOTAL NO. OF ABNORMALITIES†	TOTAL NO. OF PLANTS	STATISTICAL DIFFERENCES*	MEAN NO. OF ABNORMALITIES PER PLANT
CONTROL				2	111	a	0.018
REGENERATION	ONE-STEP			20	212	b	0.094
REGENERATION	TWO-STEP	SHORT TERM	INTACT	13	57	c	0.228
REGENERATION	TWO-STEP	SHORT TERM	PEELED	26	83	c	0.313
REGENERATION	TWO-STEP	LONG TERM	LEAF	12	45	c	0.267
REGENERATION	TWO-STEP	LONG TERM	HYPOCOTYL	44	27	d	1.630
REGENERATION	TOTAL excluding control			108	424	-	-

† excluding fasciation and changes associated with polyploidy

* a/b $\chi^2 = 5.96$, $0.01 < P < 0.05$; a/c $\chi^2 = 23.35$, $P < 0.001$; a/d $\chi^2 = 208.46$, $P < 0.001$; b/c $\chi^2 = 15.35$, $P < 0.001$; b/d $\chi^2 = 92.84$, $P < 0.001$; c/d $\chi^2 = 41.61$, $P < 0.001$

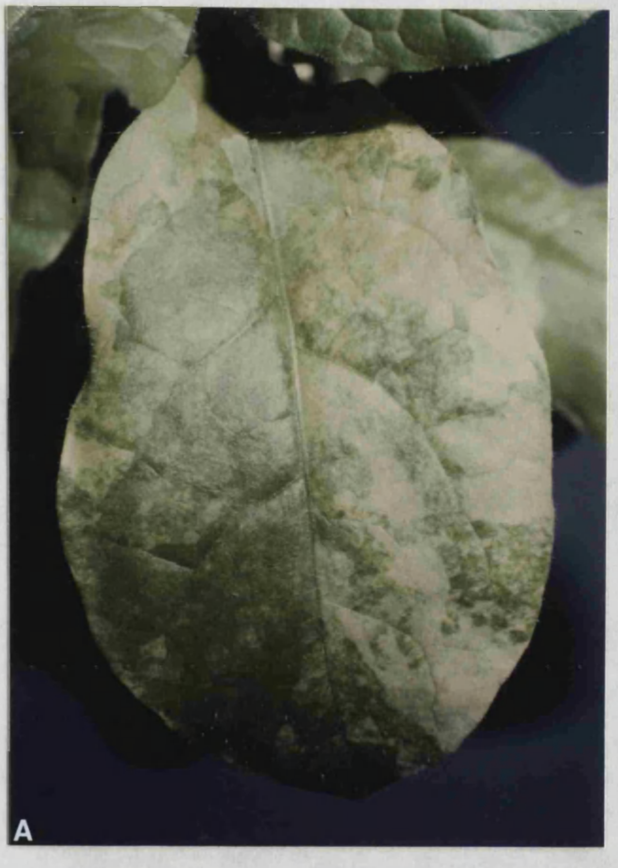
TABLE 5. Inheritance testing of variable traits

100 progeny from self-fertilisation of each of eleven variant plants were screened for the trait in question to determine if there was a genetic basis for the variation.

PHENOTYPE OF REGENERATED PLANTS	INHERITANCE
dense hairs	
long hairs	not inherited
variegated	not inherited
yellow-green	not inherited
cupped leaves	not inherited
fasciated (1)	not inherited
fasciated (2)	not inherited
fasciated (3)	not inherited
fasciated (4)	not inherited
long 'wings' running from leaf down stem	not inherited
small flowers	all progeny had small flowers

Plate 8: Possible chimaerism in regenerated plants

(A): Variegated leaf phenotype (× 0.85)
(B): "Cupped leaf" phenotype (× 0.73)



1.3.2.1.2 Fasciation

Fasciation is a change from the normal, cylindrical stem to a flatter one; the apical meristem becomes linear or comb-like, and may develop numerous growing points (White, 1916). Fasciation was observed in plants regenerated from all culture systems, but not in control seed-grown plants (Table 3). In some cases, the stem was apparently normal in shape (i.e. cylindrical) but had more than the usual number of leaves per node until the stem split into two or more parts. In other cases, the stem was much flattened and had multiple growing points at the tip. Leaf abnormalities seemed to arise when two leaves grew from nearby points on the stem and fused. The severity of the malformations was variable, ranging from branching of stems and fusion of leaves to stem distortion and breakage, and gross floral abnormalities (Plates 9 and 10).

The plant shown in Plate 10 had extremely abnormal flowers, with a flat, multi-lobed pistil and many anthers. Such linear expansion of an organ is a typical manifestation of fasciation, as is the increase in the number of elements in a whorl (White, 1916). It can be seen from this photograph that this flower also has two layers of sepals, and no petals. The transformation of the petals into sepals (a process known as **sepalody**) is uncommon and has not been previously reported in tobacco, according to Meyer (1966). In her comprehensive review, she notes that such floral abnormalities can be induced by a number of agents, including mutagens. White (1916) noted only a few instances of petalody and pistillody (that is, transformation of parts of other whorls to petals or pistils respectively), and no sepalody, amongst thousands of flowers in a genetically fasciated variety of *Nicotiana tabacum*. It is possible that this sepalody had a genetic basis and that it was unrelated to the fasciation. The inheritance of this trait could not be tested because the flowers produced no pollen or seed.

In his review, White (1916) describes the two theories concerning the origin of fasciation. That of Moquin-Tandon stated that fasciation was the result of the change in structure of a single meristem to give a flattened growing point, while that of Linné (the "concrescence" theory) attributed fasciation to the fusion of buds that grew physically close together. Today, the latter phenomenon, called **connation**, is seen as distinct from the former, "true" fasciation (Klekowski, 1988).

Fasciation can occur in response to a wide range of environmental factors (including infection by pathogens, and ionising radiation) (Klekowski, 1988) and in some cases may have a genetic basis (for

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Plate 9: Fasciation in plants from one-stage regeneration

Fasciation effects on (A) the flower (× 1.7), (B) the stem (× 0.55) and (C) the leaves (× 0.38) of *Nicotiana tabacum* plants regenerated from a one-stage tissue culture system.


A
B
C
NORMAL
GD4

facing page 48

Plate 10:

Multiple abnormalities in flowers of a fasciated plant. The petals are absent and there are two whorls of sepals (S_1 and S_2).
Each flower has many, malformed anthers and a flat multi-lobed pistil


S_1
S_2

example, fasciation of the pea variety *Pisum sativum umbellatum* was one of the seven original traits investigated by Mendel), so the fasciation observed in the regenerated plants may have had more than one cause. In the cases where inheritance was examined, there was no evidence of any genetic change (see Table 5). More extensive investigation of the fasciated trait would be required to be certain that, in this case, there was no genetic change, since the penetrance (i.e. presence or absence of expression of a mutation) of genetically determined fasciation has been found to be dependent on environmental factors (White, 1916). The widespread nature of the incidence of fasciation in my regenerated plants is, however, suggestive of a non-genetic cause.

It can be seen from Table 3 that fasciation occurred at a much higher frequency in plants from one-step regeneration than in those from two-step regeneration, despite the fact that plants from the latter system were more variable in all other aspects. In order to analyse this phenomenon further, a more detailed account of the occurrence of fasciation is given in Table 6. This shows that two batches of plants from one-step regeneration (batches 1 and 2) had much higher frequencies of fasciation than the other two batches, which were not significantly different from plants from two-step regeneration: fasciation occurred in a total of 64.86% of cultures in batches 1 and 2, compared to only 10% of all other cultures. The only apparent difference between one-step culture batches 1+2 and batches 3+4 was in the number of plants regenerated per culture. Although the number of *shoots* per culture cannot be exactly determined, as only some shoots were transferred to rooting medium and not all of these rooted and grew to maturity, an estimation can be made from the number of mature plants obtained per culture. This figure is given in the last column of Table 6. There is a high correlation coefficient ($r=0.883$) between this number and the percentage of fasciated plants produced. This shows that fasciation is more likely to arise in cultures with a higher organogenic response. I therefore propose that fasciation arises when shoot meristems are initiated very close together in the explant, and subsequently fuse. Histological examination might help in determining whether such fusion occurred.

There is some evidence that fasciation can be induced by cytokinins. For example, the symptoms of "leafy gall" (*Corynebacterium fascians* infection)— swelling of internodes and a release of lateral buds from apical dominance— can be mimicked by the application of kinetin (Thimann and Sachs, 1966). Anderson *et al.* (1982) reported an abnormality in micropropagated strawberry plants that

TABLE 6. Occurrence of fasciation

PLANT SOURCE	BATCH	TOTAL NO. OF PLANTS	NUMBER OF FASCIATED PLANTS	% FASCIATED PLANTS	*	TOTAL NO OF CULTURES	NO. OF CULTURES PRODUCING FASCIATED PLANTS	% CULTURES PRODUCING FASCIATED PLANTS	*	MEAN NO. OF PLANTS PER CULTURE †
ONE-STEP REGENERATION	1	116	25	21.6	a	24	15	62.5	d	4.83
	2	55	14	25.5	a	13	9	69.2	d	4.23
	3	15	1	6.7	b	8	1	12.5	e	1.88
	4	170	12	7.1	b	67	10	14.9	e	2.53
TWO-STEP REGENERATION	1	68	5	7.4	b	20	1	5.0	e	3.40
	2	25	2	8.0	b	16	2	12.5	e	1.56
	3	26	1	3.8	b	16	1	6.2	e	1.63
	4	12	0	0	b	7	0	0	e	1.71
	5	15	0	0	b	8	0	0	e	1.38
CONTROL		132	0	0	c	NOT APPLICABLE				

* numbers with different letters appended are significantly different with the following probabilities :

a/b $\chi^2 = 29.51, P < 0.01$
a/c $\chi^2 = 34.59, P < 0.01$
b/c $\chi^2 = 8.80, P < 0.01$
d/e $\chi^2 = 50.41, P < 0.05$

they called "multi-apexing", where leaf arrangement was disturbed by the formation of multiple apices. This phenomenon occurred in plants from cultures that had been exposed to relatively high concentrations ($\geq$ 1μM) of BAP, and could be reduced or prevented by the addition of GA_3 to the medium. It has also been observed that *Gerbera* shoots rapidly lose their apical dominance *in vitro*, becoming fasciated and multi-apexed (Blakesley, D., pers. comm.). The ability of cytokinins to release apical dominance is the basis for their use in the multiplication phase of meristem culture (Hu and Wang, 1983). 1985 in refs.

If such direct effects of the cytokinins are the cause of the fasciation observed, though, it is difficult to explain why the frequencies of variation showed such striking differences between different culture systems, even though the regeneration medium was identical in all cases. It is possible that hormonal interaction could occur: for example, the period on callus induction medium might reduce the sensitivity of the explant to BAP. The observed correlation between the number of shoots produced per culture and the extent of fasciation may not have been causal: one possible alternative explanation could be that the endogenous hormone levels varied between cultures, and influenced both the capacity for regeneration and the susceptibility to fasciation.

Direct regeneration seems to be a rapid method of producing many fasciated plants, which are generally rare in wild and cultivated plant species (White, 1916). Tissue culture could thus be a useful way of analysing this interesting plant developmental abnormality.

3.3.2.2 Quantitative traits

The following measurements were taken from mature plants regenerated from the protocols described in Section 2.2.1 and from 132 control seed-grown 'Xanthi' plants:

(i) plant height from crown to shoot tip

(ii) number of fully expanded leaves

(iii) length and width of the first 3 true leaves

From the ratio of (i) and (ii), the mean internode length was calculated, and the ratio of leaf length and width was taken as a measure of leaf shape.

First, the data were divided into two groups, those from 'normal' plants and those from plants with 'high chromosome number', based on the morphological criteria described in Section 3.3.1. The results are shown in Table 7.

Next, the data from the plants categorised as 'normal' were subdivided according to the origin of the plants . These results are shown in Table 8.

3.3.2.2.1 HEIGHT

Statistical comparison of the heights of all 'normal' plants with those of all plants with high chromosome number (Table 7) shows that plants with high chromosome number are significantly shorter than the normal plants. From Table 8, it can be seen that there are also statistically significant differences in height between plants from different sources. These differences are illustrated in Figure 4. Plants from the one-step regeneration system are significantly taller than control plants, and plants from the two-step system are significantly shorter than both the controls and the plants from the one-step system. Since inheritance was not tested for each individual tall or short plant, some of this variation may have been genetic. The entire population shift suggests, though, that physiological effects of the culture system— for example, abnormal hormone levels, may have caused the variation.

3.3.2.2.2 INTERNODE LENGTH

Internode length shows similar trends to height (Tables 7 and 8, Figure 4). Plants with high chromosome number have a significantly shorter internode length than the rest of the regenerated plants. Among the normal plants, the plants produced by one-step regeneration were not significantly different from the controls, whereas plants from two-step regeneration had a shorter mean internode length.

3.3.2.2.3 LEAF LENGTH

From Table 7, it can be seen that plants with high chromosome number have shorter leaves than other regenerated plants. The differences between different batches of "normal" plants are less marked, though, with only the long-term, hypocotyl-derived plants having significantly shorter leaves than the controls (Table 8).

3.3.2.2.4 LEAF SHAPE

Leaf length and width were highly correlated in all groups of plants, and there were no significant differences in the leaf length to width ratio between groups of "normal" cultures (data not shown).

TABLE 7. Quantitative traits in plants of different chromosome constitution

Plant height, mean internode length, leaf length and leaf length to width ratio are compared for regenerated plants classed as having normal or high chromosome numbers.

		PLANT CATEGORY	
		NORMAL	HIGH NUMBER OF CHROMOSOMES
HEIGHT (mm)	n	253	61
	mean	853.38	566.67
	sd	269.11	299.28
	*	a	b
INTERNODE LENGTH (mm)	n	251	61
	mean	34.88	25.58
	sd	10.08	10.07
	*	c	d
LEAF LENGTH (mm)	n	580	112
	mean	192.33	174.70
	sd	34.37	36.48
	*	e	f
LEAF LENGTH TO WIDTH RATIO	n	242	50
	mean	1.935	1.868
	sd	0.163	0.142
	*	g	h

* significance values: a/b $t = 7.30$, $P < 0.001$; c/d $t = 6.46$, $P < 0.001$; e/f $t = 4.99$, $P < 0.001$

TABLE 8. Effect of culture regime on quantitative traits

The heights, internode lengths, leaf lengths and leaf length to width ratios of regenerated plants classed as having normal chromosome numbers were measured. This table shows the variation in these traits that occurs in plants regenerated from different culture regimes.

PLANT SOURCE			HEIGHT (mm)				MEAN INTERNODE LENGTH (mm)				MEAN LEAF LENGTH (mm)			
			n	mean	sd	*	n	mean	sd	*	n	mean	sd	*
SEED-GROWN			163	886.36	241.03	a	129	37.37	9.20	d	149	196.05	25.37	f
ONE-STEP REGENERATION			141	958.18	257.60	b	140	39.47	9.56	d	368	193.81	31.37	f
TWO-STEP REGENERATION	SHORT-TERM	INTACT	39	745.13	203.91	c	38	30.46	7.24	e	81	196.73	41.39	f
		PEELED	61	715.39	232.46	c	61	28.90	7.44	e	110	191.15	36.12	f
	LONG-TERM	LEAF	33	722.45	189.43	c	33	30.45	6.07	e	93	145.22	31.12	g
		HYPOCOTYL	12	675.25	229.03	c	12	25.58	7.29	e	21	155.43	24.96	g

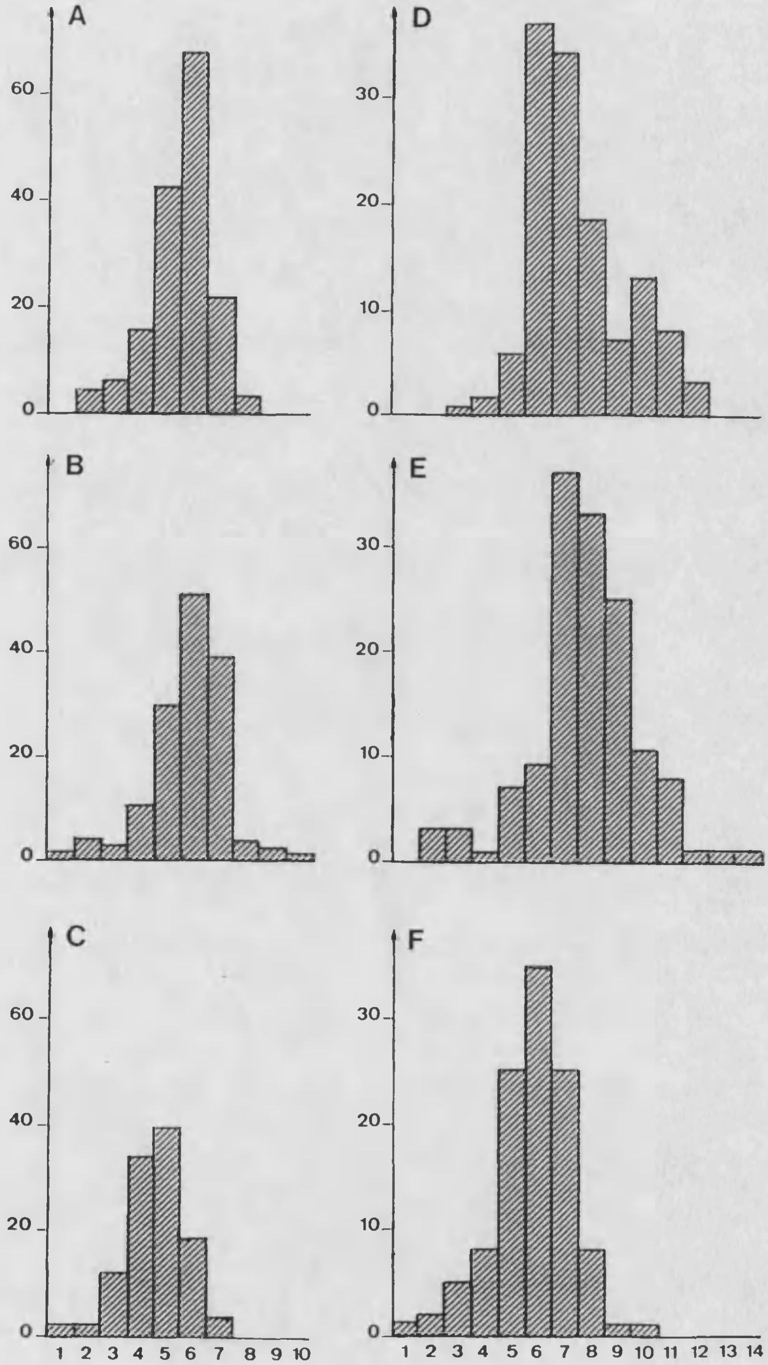
* significance values: a/b $t= 2.51, 0.01 < P < 0.02$; b/c $t= 7.72, P < 0.001$; a/c $t= 7.88, P < 0.001$; d/e $t= 13.50, P < 0.001$; f/g $t= 14.23, P < 0.001$

facing page 55

Figure 4:

Left: Frequency distribution of height of (A) control, seed-grown plants, (B) plants regenerated from one-stage culture and (C) plants regenerated from two-stage culture. *Intervals:* (1) up to 100mm, (2)101-300mm, (3) 301-500mm, (4) 501-700mm, (5) 701-900mm, (6) 901-1100mm, (7) 1101-1300mm, (8) 1301-1500mm, (9) 1501-1700mm and (10) 1701-1900mm.

Right: Frequency distribution of mean internode length of (D) control, (E) plants regenerated from one-stage culture and (F) plants regenerated from two-stage culture. *Intervals:* (1) 2.6-7.5mm, (2) 7.6-12.5mm, (3) 12.6-17.5mm, (4) 17.6-22.5mm, (5) 22.6-27.5mm, (6) 27.6-32.5mm, (7) 32.6-37.5mm, (8) 37.6-42.5mm, (9) 42.6-47.5mm, (10) 47.6-52.5mm, (11) 52.6-57.5mm, (12) 57.6-62.6mm, (13) 62.6-67.5, (14) 67.6-72.5mm.


A
B
C
D
E
F
0
20
40
60
10
30
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10 11 12 13 14

This indicates that leaf shape is highly conserved and that it is not affected significantly by the culture procedure. From Table 7, though, it can be seen that the leaf length:width ratio does vary between plants of different chromosome constitution — the plants with high chromosome number have a significantly lower leaf length to width ratio, i.e. the leaves are more rounded than usual. This rounded leaf characteristic was also noted by Murashige and Nakano (1966) but mine is the first statistical analysis.

3.3.3 Origin of chromosomal variation

A statistical comparison was made of the frequencies of plants with high chromosome number obtained from different culture systems. The results are shown in Tables 9 and 10.

It can be seen that high chromosome numbers were found in the plants regenerated from the rapid, one-step culture system, suggesting that chromosome number variation exists early on in culture. This variation could have already been present in the explant, or could have been induced early on in culture.

A comparison of tables 9 and 10 gives some insight into the origin of chromosomal changes. In the "intact" short-term cultures and the hypocotyl-derived long-term cultures, the *percentages of cultures* that produce plants with high chromosome numbers is not significantly greater than that found in one-step regeneration (Table 10), whereas the *percentage of plants* produced that have high chromosome numbers *is* significantly higher in the former (Table 9). This suggests that rather than causing the chromosomal abnormalities *de novo*, the different culture regimes influence the genetic composition of the culture indirectly, for example by stimulating the growth and division of different cell types.

In the "peeled" short-term cultures, though, the percentage of cultures producing plants with high chromosome numbers was significantly higher than that in all other culture systems. Comparison with hypocotyl the "intact" short-term cultures, which were identical to the "peeled" ones except for the presence of the lower epidermis, suggests that removal of the lower epidermis leads to a higher level of high chromosome numbers. Why should this be so? Three hypotheses are suggested:

i. a far greater level of wounding could occur in the "peeled" explants, this may induce more variability

ii. the internal cells of the explant are effectively in contact with the medium for longer in "peeled" explants, and may undergo more divisions in the same length of time; any direct or indirect effects of the medium will therefore be increased

iii. removal of the lower epidermis could cause a different set of cells to respond to callus induction, leading to a different initial genetic make-up of the culture.

The first two hypotheses imply that it is the culture process itself that induces variation, whereas the third requires that chromosome number variability exists in the explant. The following experiment was therefore carried out to determine if such variability does exist.

1.3.4 DNA content estimation

Protoplasts were isolated from the mesophyll and the midrib of mature, fully expanded *Nicotiana tabacum* described in Section 3.2.2.1. They were used immediately for DNA content estimation (Section 3.2.2). Feulgen-stained samples fade gradually when exposed to light, so the absolute absorbance reading is irrelevant, and must be corrected in some way to obtain data that can be compared meaningfully. For this reason, a background reading was taken for each cell of the absorbance of the cytoplasm. This should correct the total reading for any absorbance due to cytoplasmic DNA and to background staining. A measure of the DNA content, in arbitrary units, was determined by subtracting the background count from the total count. These are the figures given in Table 11. It must be pointed out that such a correction is only valid if the Feulgen displays a linear degradation pattern; if, instead, the stain decays exponentially, a correction factor based on the *ratio* of the background and the actual counts would be more appropriate. Both correction methods gave figures for DNA content that were correlated with nuclear size; the significance of the correlation was higher for the linear correction method. Statistical analysis also showed that data corrected by the linear decay model had a more normal distribution than data corrected by an exponential model. The former data have therefore been presented here. It must be noted that the actual DNA content cannot be determined since standard diploid plant tissue of known DNA content was not available.

Cell and nuclear size were also determined, and all data are shown in Table 11. The distributions of the data are illustrated by histograms in Figure 5.

TABLE 9. Chromosome constitution of plants from different culture regimes (I)

PLANT SOURCE				TOTAL NO. OF PLANTS	NO. OF PLANTS WITH HIGH NO. OF CHROMOSOMES	% PLANTS WITH HIGH NO. OF CHROMOSOMES	*
SEED-GROWN				132	0	0	-
ONE-STEP REGENERATION			Batch 1	116	21	18.96	a
			2	55	8	14.55	a
			3	170	21	12.35	a
TWO-STEP REGENERATION	SHORT TERM	INTACT	Batch 1	25	6	24.00	b_1
			2	26	2	7.69	b_2
			3	12	6	50.00	b_1
		PEELED	Batch 1	68	21	30.88	b_1
			2	15	7	46.67	b_1
			3	12	7	58.33	b_1
	LONG TERM	LEAF	Batch 1	23	6	26.09	a
			2	37	8	21.62	a
		HYPOCOTYL	Batch 1	2	2	100.00	c
			2	30	25	83.33	c

* significance values: b_1/b_2 $\chi^2 = 7.99$, $0.001 < P < 0.01$; a/b $\chi^2 = 16.04$, $P < 0.001$; a/c $\chi^2 = 67.90$, $P < 0.001$; b/c $\chi^2 = 31.57$, $P < 0.001$

TABLE 10. Chromosome constitution of plants from different culture regimes (II)

PLANT SOURCE				TOTAL NO. OF CULTURES	NO. OF CULTURES PRODUCING PLANTS WITH A HIGH NO. OF CHROMOSOMES	% OF CULTURES PRODUCING PLANTS WITH A HIGH NO. OF CHROMOSOMES	*
SEED-GROWN				-	-	-	-
ONE-STEP REGENERATION			Batch 1	24	14	58.33	d
			2	13	5	38.46	d
			3	67	22	32.83	d
TWO-STEP REGENERATION	SHORT TERM	INTACT	Batch 1	16	5	31.25	d
			2	16	2	12.50	d
			3	6	3	50.00	d
		PEELED	Batch 1	20	9	45.00	e
			2	8	5	62.50	e
			3	8	6	75.00	e
	LONG TERM	LEAF	Batch 1	n/a	n/a	n/a	-
			2	n/a	n/a	n/a	-
		HYPOCOTYL	Batch 1	2	2	100.00	d
			2	7	7	100.00	d

* significance value: d/e $\chi^2 = 3.96$, $0.05 < P < 0.01$

n/a data not available

TABLE 11. Cell size, nuclear size and DNA contents of tobacco leaf cells

The diameter and nuclear size of freshly isolated protoplasts from the mesophyll and midrib of *Nicotiana tabacum* leaves are shown, with the DNA content as estimated by Feulgen microdensitometry.

		PROTOPLAST SOURCE	
		LEAF MESOPHYLL	MIDRIB
PROTOPLAST	n	201	189
DIAMETER	mean	30.51	43.73
(µm)	sd	9.86	26.26
	*	a	b
NUCLEAR	n	153	140
SIZE	mean	58.87	78.16
(epu^2)†	sd	26.33	46.52
	*	c	d
	n	153	140
DNA	mean	27.25	29.79
CONTENT	sd	9.57	11.36
(U)††	*	e	f
CORRELATION OF DNA CONTENT WITH NUCLEAR SIZE‡		0.568	0.620

† 1 epu = 80µm (the third dimension of the nucleus, i.e. the distance between the slide and the coverslip, was not calculated)
†† arbitrary units
‡ linear correction model (see text)
the significance of the correlations are t= 8.48 and 9.28 for mesophyll and midrib respectively ($P < 0.001$ in each case)

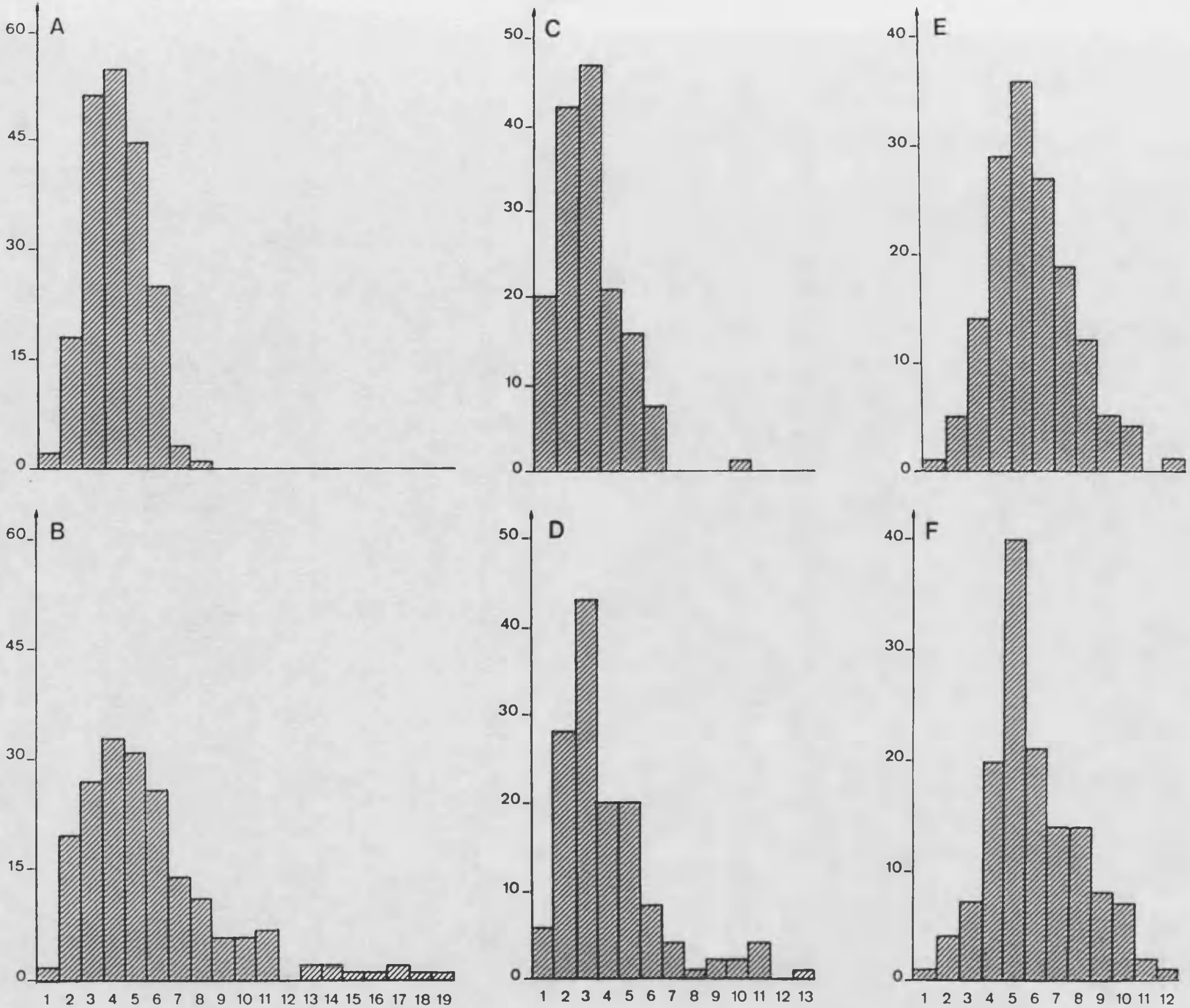
* significant differences: a/b t=9.08, $P < 0.001$; c/d t=4.42, $P < 0.001$; e/f t=2.09, $0.02 < P < 0.05$

Figure 5: Cell size, nuclear size and DNA content of tobacco leaf cells

Left: Frequency distribution of diameter (in e.p.u., 1 e.p.u.= 80μm) of freshly isolated protoplasts from (A) leaf mesophyll and (B) leaf midrib. *Intervals:* (1) 0.050-0.149, (2) 0.150-0.249, (3) 0.250-0.349, (4) 0.350-0.449, (5) 0.450-0.549, (6) 0.550-0.649, (7) 0.650-0.749, (8) 0.750-0.849, (9) 0.850-0.949, (10) 0.950-1.049, (11) 1.050-1.149, (12) 1.150-1.249, (13) 1.250-1.349, (14) 1.350-1.449, (15) 1.450-1.549, (16) 1.550-1.649, (17) 1.650-1.749, (18) 1.750-1.849, (19) 1.850-1.949.

Centre: Frequency distribution of nuclear size (in μm^2) of freshly isolated protoplasts from (C) leaf mesophyll and (D) leaf midrib. *Intervals:* (1) 10-29.9, (2) 30-49.9, (3) 50-69.9, (4) 70-89.9, (5) 90-109.0, (6) 110-129.9, (7) 130-149.9, (8) 150-169.9, (9) 170-189.9, (10) 190-209.9, (11) 210-229.9, (12) 230-249.9, (13) 250-269.9.

Right: Frequency distribution of nuclear DNA content (in arbitrary units) of freshly isolated protoplasts from (E) leaf mesophyll and (F) leaf midrib. *Intervals:* (1) 2.5-7.4, (2) 7.5-12.4, (3) 12.5-17.4, (4) 17.5-22.4, (5) 22.5-27.4, (6) 27.5-32.4, (7) 32.5-37.4, (8) 37.5-42.4, (9) 42.5-47.4, (10) 47.5-52.4, (11) 52.5-57.4, (12) 57.5-62.4.


A
B
C
D
E
F
0 15 30 45 60
0 10 20 30 40 50
0 10 20 30 40
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
1 2 3 4 5 6 7 8 9 10 11 12 13
1 2 3 4 5 6 7 8 9 10 11 12

Cell size was much more variable in the midrib (standard deviation = 26.26) than in the leaf mesophyll (standard deviation = 9.86). This is consistent with the relative diversity of cell types in the midrib compared to the mesophyll. Nuclear size was significantly greater in the midrib cells, but although there was a significant correlation between nuclear size and DNA content (correlation coefficients r = 0.568 and r = 0.620 for the mesophyll and midrib respectively), the difference in DNA content between the two tissue types was only significant at fairly low levels.

Most nuclei in the mesophyll cells were approximately spherical, with 9.2% being elongated, whereas 25% of midrib cells had irregularly shaped nuclei. Various abnormal nuclear shapes were observed only in midrib cells, including "U"-shapes, dumb-bell shapes and nuclei with fissures (Plate 11). The differences between the two tissues in the numbers of abnormally shaped nuclei are significant (χ^2=13.22, $P < 0.01$). The DNA content of the aspherical nuclei was found to be significantly greater than that of spherical nuclei (see Table 12). This is in agreement with the conclusions of Brossard (1975); she found an association between high ploidy and nuclear shape irregularity in *Nicotiana tabacum* cells. She also observed, though, that cambial cells tended to have elongated nuclei, so the increased number of aspherical nuclei that I found in the leaf midrib could be at least partly explained by the presence of cambial cells in this tissue.

The question of whether these abnormally shaped nuclei are polyploid or not can be addressed by studying their DNA content values: In an entirely diploid tissue, cells that were in the process of DNA replication would have DNA contents between 2C and 4C, while any cells that were resting in G_2 (i.e. the post-synthesis phase) would have a 4C content. The range of DNA contents would therefore be from 2C to 4C, and the ratio between the maximum and minimum DNA contents in the tissue would not be greater than 2. In Table 13, the distributions of DNA content in tobacco leaf mesophyll and midrib cells are described. It can be seen that the ratio of the maximum to the minimum readings of DNA content is much greater than 2 in both tissue types, suggesting that neither tissue is wholly diploid. This ratio could appear higher than it really is if there was a high error in DNA content counting, so it would be important to use a reference sample of known DNA content to determine whether there is such a high error. These preliminary findings do indicate, though, that both of these tissues contain non-diploid cells. Without knowing the actual values to which the arbitrary DNA content units correspond, it is not possible to decide whether the non-diploid cells have more

Plate 11:

Elongated, fissured nuclei from the root tip of a regenerated plant with approximately 65 chromosomes (× 3000)

TABLE 12. Nuclear shape and its relationship to DNA content

Nuckei were classified as spherical or irregularly shaped; the table below shows the relationship between nuclear shape and DNA content

NUCLEAR SHAPE	DNA CONTENT (U)†		
	n	mean	sd
NORMAL	247	27.48	9.97
IRREGULAR	46	33.86	11.80

† arbitrary units
significance: t= 3.88 , P < 0.001

TABLE 13. DNA content distribution in different tissues

DNA CONTENT DISTRIBUTION	TISSUE	
	MESOPHYLL	MIDRIB
MINIMUM	7.70	7.40
LOWER QUARTILE	20.70	22.73
MEDIAN	26.00	27.00
UPPER QUARTILE	33.70	36.23
MAXIMUM	60.00	58.50
RATIO OF MAXIMUM TO MINIMUM	7.79	7.91

than a 4C DNA content or less than a 2C content. Again, a standard reference sample would be needed to distinguish these two possibilities. The broad range of DNA content values in the midrib cells, and the high proportion of aspherical nuclei in these cells suggests that there may be endopolyploidy in this tissue. This is in agreement with the results of Brossard-Chriqui (1980), which showed that there were cells with up to the 8C DNA content in *Nicotiana tabacum* 'Xanthi' leaves, especially in the parenchyma and collenchyma of the vein. Since the actual DNA content values are not known, part of the explanation for the large range in DNA contents could be that some cells had less than the diploid DNA content. Loss of DNA has been reported during the senescence of tobacco leaves (Dhillon and Mikshe, 1981). Since callus was often observed to arise from leaf veins (Section 2.2.2), polyploid cells in the vascular tissue of the explant could take part in callus formation and could thus have been the origin of the regenerated shoots with high chromosome number. de Boucaud *et al.* (1985) concluded that newly formed *Nicotiana tabacum* callus retained the same distribution of DNA contents as the explants (leaf parenchyma and stem sections), suggesting that abnormal cells *in vivo* can participate in callus induction. In contrast, Brossard-Chriqui (1980) found that roots regenerated directly from tobacco leaf veins were entirely diploid.

Since my results provide evidence that the explant contains some polyploid cells, hypothesis (iii) above cannot be ruled out. Further work on DNA content analysis, including quantification of DNA content in different explants and during the course of callus induction and plant regeneration, would be necessary to assess the relative importance of factors such as explant source and culture regime in determining genetic stability *in vitro*.

1.4 Summary

Tetraploid plants and aneuploids at the tetraploid level were regenerated from culture and their high chromosome number was associated with characteristic morphology; alterations in floral appearance and fertility were particularly striking. Aneuploidy at the diploid level and mixoploidy also occurred in regenerated plants, but generally had little apparent effect on phenotype.

The morphological criteria described were used to estimate the frequency of "high chromosome number", that is, polyploidy, or aneuploidy at the polyploid level, in different culture systems.

Different culture systems varied significantly in the percentage of plants produced with high chromosome number, but the duration of culture (from 8 weeks to 4 months) did not affect the percentage of cultures that produced plants with high chromosome numbers. The only factor that significantly increased this latter statistic was the removal of the lower epidermis of leaf explants prior to culture. These findings, together with DNA content counts of leaf tissue, are consistent with the hypothesis that chromosome number variation exists within plants *in vivo* and that this variation contributed to the observed somaclonal variation.

Variation in quantitative traits occurred both between ''normal'' regenerated plants and those with high chromosome number and between plants regenerated from different culture systems. Variation also occurred in qualitative traits. The inheritance of eleven variable traits was examined; a ''small flower'' phenotype was transmitted to all progeny following self-fertilisation— these results are consistent with the suggestion that this was a homozygous mutation. The remaining traits were not inherited. Whereas the extent of phenotypic variation in general increased with increasing culture duration, the frequency of one trait, fasciation, was greatest in plants from one-step regeneration. It was found to be correlated with the number of plants regenerated from each culture.

4. DETECTION OF GENETIC VARIATION USING RFLP ANALYSES

4.1 Introduction

4.1.1 Maximising the chances of detecting RFLPs

Beckmann and Soller (1986a) calculate that only 1 in 100 point mutations will be detected as a RFLP, even when a large number of restriction enzymes is tested. If the chance of detecting a RFLP is to be maximised, the factors that affect this chance must be considered:

4.1.1.1 Nature of probe

Probe copy number: The major application of RFLPs to date has been their use as new markers for genetic mapping. For this purpose, single- or low-copy number sequences have been selected, since these will tend to detect single loci. In cultivar or parentage analysis, though, many loci may have to be screened (Soller and Beckmann 1983) for conclusive identification. The number of individual probes needed to distinguish two individuals could be reduced if multi-locus probes were available. Jeffreys *et al.* (1985) identified such sequences by using low stringency hybridisation conditions to detect related sequences. Different classes of sequences may have different rates of genetic change. Tandemly repeated sequences, for example, seem to be particularly prone to changes in copy number (Flavell, 1986), whereas single-copy sequences, which are likely to be functional genes, may be highly conserved.

Probe length: The longer a probe, the longer is the region of DNA that will be picked out on a Southern blot. Although increasing the length of the probe will thus increase the length of DNA screened and therefore improve the chances of detecting RFLPs, it will also make it more likely that the probe will consist of more than one type of sequence class. Such a 'compound' probe would produce a complex pattern and so some authors have chosen to limit probe length to 1kb (Landry and Michelmore, 1985).

Source of probe: Probes can either come from genomic or cDNA libraries. cDNA clones necessarily represent only the transcribed fraction of the genome, whereas genomic clones derive from more of the genome and could therefore be considered as more 'representative'. It might be expected that cDNA clones would detect less variation than genomic clones since transcribed sequences are thought to be under more sequence constraint than other sequences. Indeed, cDNA clones detected less RFLPs than genomic clones in human DNA (Helentjaris and Gesteland, 1983). However, Helentjaris *et al.* (1985) found similar levels of polymorphism with both types of probes in maize, and Landry *et al.* (1987) found cDNAs more useful for detecting RFLPs in lettuce. One disadvantage of cDNA clones is that they may not always give an intense signal when hybridised to genomic DNA (Helentjaris, 1987), possibly because the cDNA lacks the introns present in the genomic DNA.

4.1.1.2 Restriction enzymes

A point mutation will result in a RFLP if it creates or destroys a restriction site. Therefore, the more actual and potential restriction sites that exist for a given enzyme, the more DNA is screened. The theoretical frequencies of any 4- and 6-base sequences (1 in 256 and 1 in 4096 respectively) suggest that enzymes with 4-base recognition sequences would be more likely to detect variation. Actual base frequencies (or dinucleotide frequencies) can be used to make a more accurate prediction of the frequencies of particular sequences. For example, since plant DNA generally has more A and T residues than C or G, enzymes with high levels of A and T in their recognition sequences may be the most useful (Wijsman, 1984). In this paper, theoretical relative efficiencies of different enzymes for detecting RFLPs in human DNA are calculated; these calculations predict that enzymes having 4bp recognition sequences will be more efficient at detecting point mutations than enzymes with 6bp recognition sites, provided that the minimum detectable fragment size is at least 200bp. The amount of polymorphism detected by a given enzyme will also depend on the mutability of its recognition sequence. Mutation rates are not the same for all bases (Brown and Clegg, 1983): for example, it has been shown that methylated cytosine residues are 'hot spots' for mutation (Coulondre *et al.*, 1978) so enzymes that contain commonly methylated sequences, such as $^{5'}$ CG $^{3'}$ and $^{5'}$ CXG $^{3'}$, may be useful for detecting RFLPs. Donis-Keller *et al.* (1986) did find more variation with *Msp*I and *Taq*I, which both contain CG pairs in their recognition sites. However, it must also be remembered that methylation may inhibit restriction digestion (see Section 4.1.1.2); this may outweigh the benefits of the increased mutability when considering the enzyme(s) to be used.

Technical considerations are also important. For example, the resolution of an agarose gel is better for small fragments, and so size changes will be more easily detectable in smaller fragments, such as those generated by restriction enzymes with 4-base pair recognition sequences. This may be an important factor when searching for small insertions and deletions (Bishop *et al.*, 1983). On the other hand, very small fragments may be undetectable, and much information will be lost if the minimum detectable fragment size is too great (Wijsman, 1984).

In practice, no one restriction enzyme seems to be consistently good at detecting RFLPs in all the systems studied. This may be due to different base frequencies between species and even between different parts of the genome. So, despite the considerations above, the selection of 'useful' restriction enzymes must still be largely a matter of trial and error.

4.2 Aims

The aims of these experiments were to assess the value of RFLP analyses in the detection and quantification of genetic variation in tobacco, and to compare the abilities of different restriction enzymes and probes to detect variation. The long-term aim was to develop a system for screening plants regenerated from tissue culture, in order to establish the frequency and nature of somaclonal variation.

4.3 Materials and methods

4.3.1 Preparation of media

Reagents were obtained from Sigma and BDH Ltd. unless otherwise stated. All solutions were made up in 'Milli-Q' water (distilled water purified by the Millipore reverse osmosis system). Where possible, all reagents and equipment were sterilised by autoclaving at 15lb/in^2 (121°C) for 15 minutes. Heat-labile substances were sterilised by filter sterilisation using 0.2μm 'Mini-Sart'® NML disposable membrane filtration units (Sartorius).

4.3.2 General recipes

EDTA:	186.1g of disodium ethylene diamine tetraacetate (dihydrate) was dissolved in water per litre of 0.5M EDTA. The pH was adjusted with NaOH. (Note: EDTA will not dissolve until the pH reaches about 8.0).
TRIS-HCL:	Trizma base (Sigma) was dissolved in water (121.1g/l) , and the pH was adjusted with concentrated HCl.
TE:	10mM Tris-HCl of required pH, 1mM EDTA pH 8.0
20× SSC:	3M NaCl, 0.3M trisodium citrate
20× SSPE:	3.6M NaCl, 0.2M $NaH_2PO_4.H_2O$, 0.02M EDTA (pH 7.7)
RNase (DNase free):	Bovine pancreatic ribonuclease (RNase A, Sigma) was dissolved at 10mg/ml in 10mM Tris-HCl (pH 7.5), 15mM NaCl. To remove DNase, the RNase was heated in a boiling water bath for 15 minutes. It was stored at -20°C.
Phenol:	Crystalline phenol (BDH) was re-distilled as described by Wallace (1987), then 8-hydroxyquinoline was added to a concentration of 1mg/ml and the phenol stored at -20°C. Thawed phenol was equilibrated against TE (pH 8.0) and then mixed with an equal volume of chloroform and 1/24 volume of isoamylalcohol. This mixture was stored at -20°C.

4.3.3 General techniques

4.3.3.1 Phenol extraction

Proteins were removed from aqueous solutions of nucleic acids by extraction with phenol or with phenol-chloroform-isoamylalcohol prepared as in Section 4.3.2. In a phenol and chloroform resistant tube, an equal volume of phenol or phenol-chloroform-isoamylalcohol was mixed with the aqueous solution to form an emulsion. The phases were separated by centrifugation at room temperature (5-10 minutes at 2000 *g*, or 1 minute in a microfuge), and the aqueous (upper) phase was transferred to a fresh tube, avoiding the protein at the interface. For small volumes and/or low concentrations of DNA, the interface was washed with TE and re-extracted to minimise loss of DNA.

4.3.3.2 Ethanol precipitation of DNA

0.1 volumes of 3M sodium acetate (pH 5.2) was added to the DNA solution, followed by 2 volumes of ethanol. The DNA/ethanol mixture was vortexed and then placed at -20°C for 1-16 hours. Following precipitation, the DNA was collected by centrifugation (5-15 minutes at high speed in a microfuge, or 20-30 minutes in a Sorvall SS-34 rotor) at 4°C. The pellet was washed in 70% (v/v) ethanol, drained and dried by spinning for 5 minutes in a Speed Vac Concentrator (Savant).

4.3.3.3 Quantification of nucleic acids

UV spectrophotometry: for pure preparations of DNA, the optical density of the sample in light of wavelength 260nm is proportional to the concentration of DNA (Maniatis *et al.*, 1982): double-stranded DNA at a concentration of 50μg/ml has an OD_{260} of 1.0. The ratio of the optical densities at 260nm and 280nm gives an indication of the purity of the sample: pure DNA has an OD_{260}/OD_{280} of 1.8. The optical density of samples was measured using a Shimadzu uv-260 spectrophotometer, with quartz cuvettes.

Estimation by ethidium bromide fluorescence: a series of concentration standards was constructed, using DNA of the same size as the DNA to be assayed: bacteriophage λ DNA, either intact or digested with a restriction enzyme, was usually used as the standard. Sample DNA was run with a range of concentrations of the standard DNA on an agarose gel in the presence of ethidium bromide. Following electrophoresis, the amount of DNA in the sample was estimated by comparison with the standards.

4.3.3.4 Restriction digestion of DNA

Restriction enzymes: Restriction enzymes were obtained from Northumbria Biologicals Ltd. (Cramlington, Northumbria) and were stored at -20°C. The recognition sites of the enzymes used are shown in Table 15.

Reaction buffers: Reaction buffers were supplied with the restriction enzymes and were stored at -20°C.

Reaction conditions: digestion reactions were set up in 0.5ml Eppendorf tubes. The following components were added, in this order:

i. water to give the final volume required
ii. 0.1 volume of 10× reaction buffer
iii. 0.1 volume of 1mg/ml BSA (Pharmacia,enzyme grade) †
iv. DNA to be digested
v. restriction enzyme *

† for long reactions only, to stabilise the enzyme

* *Note*: One unit of a restriction enzyme is defined as that amount of enzyme required to cleave 1µg of DNA in one hour in optimal reaction conditions. While such DNA to enzyme ratios and reaction times are adequate for complete digestion of bacteriophage λ DNA, longer incubation times and higher enzyme concentrations are frequently necessary for the complete digestion of plant DNA. Precise details of individual reaction times are given in the relevant protocols.

The contents of the tube were mixed gently, then spun down briefly in a microfuge. All digests were carried out at 37°C except for those using *Bst*UI (60°C) *Sma*I (25°C), and *Taq*I (65°C under paraffin oil to prevent evaporation).

Terminating the reaction: Reactions were terminated by heating the samples at 65°C for 5 minutes. For restriction enzymes that are not completely inactivated by heat treatment (e.g. *Hin* dIII and *Taq* I, the DNA was phenol extracted and ethanol precipitated.

4.3.3.5 Gel electrophoresis

Preparation of agarose gels: agarose (Sigma type I, low EEO) to give the required concentration was dissolved in 1× TBE buffer by heating gently over a Bunsen burner until the solution was completely clear. The solution was allowed to cool to 55°C, then ethidium bromide was added to a concentration of 500ng/ml before pouring into a gel casting mould. Generally, 0.8% (w/v) agarose gels were used for DNA digested with restriction enzymes with 6 base pair recognition sites, while 1.2% (w/v) gels were used for DNA digested by enzymes with 4 base pair recognition sequences. When the gel had set, the well-forming comb was removed, and the gel was transferred into a gel electrophoresis tank, where it was just covered with 1× TBE (containing 500ng/ml ethidium bromide). A stock solution of ethidium bromide (BDH) at 10mg/ml in water was stored at 4°C.

10× TBE: 0.89M Tris-borate, 0.89M boric acid, 0.25M EDTA

Preparation of DNA samples for gel electrophoresis: one tenth volume of 10× loading buffer (see below) was added to each sample and was mixed in gently. The samples were then incubated at 65°C for 5 minutes, cooled briefly on ice and then loaded into the wells.

Loading buffers: 10× loading buffer consisted of 50% (v/v) glycerol, 0.25% (w/v) dye— either bromophenol blue or orange-G— and 100mM EDTA (pH 8.0). In agarose gels, the bromophenol blue marker migrates with DNA molecules of approximately 500bp, while orange-G (7-hydroxy-8-phenylazo-1,3-naphthalenedisulphonic acid) migrates near the front.

Molecular weight markers: molecular weight markers were included on each gel. DNA from bacteriophage λ strain cI857 Sam I was obtained from Northumbria Biologicals Ltd.. The λ DNA was digested with either *Hin* dIII or *Hin* dIII and *Eco* RI (Section 4.3.3.4). Fragment sizes are shown in Table 14.

Electrophoresis: the gel electrophoresis tank was connected to a LKB 2197 power supply. Unless otherwise stated, electrophoresis was at 1V/cm overnight. The migration of the DNA was followed using the loading buffers, which co-migrate with DNA of particular sizes (see above).

TABLE 14. Sizes of λ DNA fragments used as molecular weight markers

λ/HindIII (bp)	λ/HindIII and EcoRI (bp)
23130*	21226*
9416	5148
6557	4973
4361*	4277
2322	3530*
2027	2027
564	1904
125	1584
	1330
	983
	831
	564
	125

Note :
The fragments marked with an asterisk contain the *cos* sites (cohesive ends) and so will tend to stick together. To separate these fragments, the digested λ DNA was heated at 65°C for 5 minutes prior to loading onto gels.

4.3.4 Bacterial strains

4.3.4.1 Sources and genotypes of strains

i. *Escherichia coli* JM109 was obtained from J.Messing. It is ampicillin sensitive and has a defective *lac*Z gene which can be complemented by a peptide induced in the presence of IPTG (see Section 4.3.5.5.4). Its genotype is: *recA* I, *endA* I, *gyrA* 96, *relA* 1, λ^-, Δ *lac-pro* A,B, [F', *tra* D36, *pro A,B*, *lac*I^q, ΔM15]

ii. *E. coli* JM83/pUC18 was obtained from Bethesda Research Laboratories. The pUC18 plasmid confers ampicillin resistance. The genotype of JM83 is *ara*, Δ(*lac-pro A,B*), *thi*, *str A*, ϕ80d *lac*Z, ΔM15.

iii. *E. coli* TB-1 was obtained from Bethesda Research Laboratories. It is a proline auxotroph and is resistant to streptomycin. It is constitutive for β-galactosidase production. Its genotype is Δ *lac-pro*, *str A*, *ara*, *thi*, ϕ80d, *lac* Z, *hsd R*.

4.3.4.2 Bacterial growth media

LB: 1% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl (pH 7.5)

SOB: 2% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 10mM $MgSO_4$ (both added after autoclaving from a 1M stock solution) (pH 6.8) *Note*: The SOB was autoclaved for 30 minutes, and cooled prior to addition of the magnesium salts.

Media were solidified, if required, by the addition of 1% (w/v) agar (Difco) If required, antibiotics were added to the cooled, autoclaved media from filter-sterilised stock solutions:

AMPICILLIN: A stock solution of 25mg/ml in water was filter-sterilised and stored at -20°C. The working concentration was 0.01% (w/v).

CHLORAMPHENICOL: A stock solution of 34mg/ml in 100% ethanol was filter-sterilsed and stored at -20°C. The working concentration was 30μg/ml for selection of resistant bacteria or 170μg/ml for amplification of plasmids.

4.3.4.3 Measurement of growth

The growth of liquid cultures was monitored by measuring the optical density in light of a wavelength of 550nm using a Pye Unicam PU 8650 spectrophotometer (Philips).

4.3.4.4 Storage of bacterial strains

4.3.4.4.1 Short-term storage

Bacteria were streaked onto LB agar plates, containing ampicillin where appropriate, then incubated, inverted at 37°C overnight. The plates were sealed with 'Parafilm' and stored inverted at 4°C for up to 6 weeks.

4.3.4.4.2 Long-term storage

Overnight cultures of bacteria in SOB (Section 4.3.4.2) were diluted 1:1 with 40% (w/v) glycerol in SOB. Aliquots of 1ml in 1.5ml Eppendorf tubes were chilled on ice, then flash-frozen in liquid nitrogen and stored at -80°C.

Up to 100 individual cultures can be grown and stored on a single 90mm Petri dish. Each genomic clone was streaked onto a square of a grid marked on a 82mm diameter nylon membrane (Amersham 'Hybond-N') on a LB+Ap agar plate. The plate was incubated, inverted at 37°C overnight, then the membrane was transferred to a fresh LB+Ap plate containing 25% (w/v) glycerol and incubated at 37°C for a further 2 hours. The plate was sealed with 'Parafilm', wrapped in a plastic bag and stored at -20°C.

4.3.5 Techniques for the analysis of plant DNA

4.3.5.1 Plant DNA extraction

4.3.5.1.1 DNA extraction using CTAB

This method was based on that of Draper *et al.* (1988). Since CTAB precipitates at temperatures below 15°C, the temperature must at no stage fall below this. Fresh leaf material was swabbed down with 70% (v/v) ethanol and rinsed with water. 10g of leaf material (excluding major veins) was frozen in liquid nitrogen in a mortar, then 4g of alumina were added and the leaf material was ground to a fine powder with a pestle. The powder was transferred to a 50ml chloroform resistant centrifuge tube, and 15ml of *hot* (95°C) 2× CTAB extraction buffer (see below) and 300μl of β-mercaptoethanol were added. The tissue and buffer were mixed well with a spatula, incubated at 56°C for 20 minutes, then allowed to cool to room temperature. The solution was extracted twice with an equal volume of chloroform/isoamylalcohol (24:1 v:v), separating the phases by centrifuging at 10000rpm (SS-34 rotor) for 10 minutes at 20°C. 0.1 volume of 10% CTAB (see below) was mixed in gently, and a third chloroform/isoamylalcohol extraction was performed. To the aqueous phase, an equal volume of 1× CTAB precipitation buffer (see below) was added. The 2 solutions were mixed well and left at room temperature to precipitate (at least 30 minutes). The precipitate was collected by centrifugation at 1500 *g* for 15 minutes, then it was drained and resuspended in 5ml 1M NaCl by incubating at 56°C. The DNA was then precipitated by the addition of 2 volumes of ethanol followed by incubation at -20°C overnight. The precipitate was collected by spinning for 5 minutes in a microcentrifuge. It was washed 3 times in 65% (v/v) ethanol and 3 times in 85% (v/v) ethanol, then dried (Section 4.3.3.2) and redissolved in 500μl sterile H_2O.

Reagents:

2× CTAB extraction buffer: 100mM Tris-HCl (pH 8.0), 1.4M NaCl, 20mM EDTA, 2% (w/v) CTAB (cetyl-trimethyl-ammonium bromide)

1× CTAB precipitation buffer: 50mM Tris-HCl (pH 8.0), 10mM EDTA, 1% (w/v) CTAB,

10% CTAB: 10% (w/v) CTAB in 0.7M NaCl

4.3.5.1.2 DNA extraction using urea extraction buffer

(Based on the method of Chen *et al.* (1986). 2g of fresh leaf tissue was frozen in liquid nitrogen, ground to a fine powder in a mortar with a pestle, then transferred into 6ml urea extraction buffer (see below) in a chloroform resistant tube (Nalgene FEP (fluorinated ethylene propylene)) and mixed thoroughly. An equal volume of phenol/chloroform (Section 4.3.2) was mixed in by shaking, and the tube was kept at room temperature for 15 minutes, mixing occasionally. The emulsion was centrifuged in a Sorvall SS-34 rotor at 8000rpm for 10 minutes at 4°C, and the aqueous phase was filtered through 60μm nylon mesh into a fresh tube. The phenol/chloroform extraction was repeated, then the DNA was precipitated by adding 1ml 4.4M ammonium acetate (see below) and isopropanol to 13 ml. The nucleic acid was recovered by centrifuging at 6000rpm for 5 minutes, and the pellet was dissolved in 500μl TE. The DNA was then reprecipitated by adding 100μl 4.4M ammonium acetate (pH 5.2) and 700μl isopropanol. The precipitate was collected by spinning at high speed in a microfuge for 30 seconds; the pellet was washed in 75% (v/v) ethanol, dried (Section 4.3.3.2) and resuspended in 200μl TE.

Reagents:

Urea extraction buffer: 7M urea, 0.313 M NaCl, 0.05M Tris-HCl (pH 8.0), 0.02M EDTA (pH 8.0), *Note*: Urea is not autoclavable. The urea was added to autoclaved stock solutions of the other components.

Ammonium acetate: Ammonium acetate was prepared by mixing 105ml H_2O with 50ml glacial acetic acid, then adding, dropwise, 45ml ammonium hydroxide (ammonia water). *Note*: This reaction is potentially explosive.

RNase treatment: Both plant DNA extraction methods described above also extract RNA. This was removed following restriction digestion of DNA by adding 1μl of 10mg/ml DNase-free RNase A (Section 4.3.2) per 50μl DNA solution, and incubating at 37°C for 5-10 minutes.

4.3.5.1.3 Purification of tobacco DNA by CsCl density gradient centrifugation

Plant DNA was extracted (Section 4.3.5.1) and made up to 10ml with water. Exactly 10ml of solid CsCl was added, followed by 1.04ml of 10mg/ml ethidium bromide. The solution was centrifuged at

50krpm in a vertical rotor (vTi65) in a Beckman L5-50 B ultracentrifuge, overnight. The DNA band was removed and purified as for plasmid DNA (Section 4.3.5.4.2).

4.3.5.1.4 Chloroplast DNA isolation

(Based on the method of Kemble (1987).) 10g leaf material was homogenised (2×5 second pulses in an 'Osterizer' blender) in 100ml buffer D (see below), filtered through 3 layers of cheesecloth and 2 layers of 60μm mesh. The homogenate was centrifuged (1000 *g* for 10 minutes), resuspended in 30ml buffer E (see below), centrifuged as before and then resuspended in 9.5ml buffer E. In a 30ml polycarbonate ultracentrifuge tube (MSE), a sucrose gradient was prepared as described by Saltz and Beckman (1981), with layers of 60% (w/v), 45% and 25% sucrose in Buffer E (the concentration of sucrose being highest at the bottom of the tube). The layers of the gradient were mixed slightly by stirring, to avoid very tight packing of the chloroplasts. The DNA extract was layered on top of the 25% sucrose and the gradient was centrifuged at 20krpm for 1 hour at 4°C in a MSE swing-out rotor. The green layer was removed, diluted gradually with buffer C (see below) with 100μg/ml Proteinase K (Protease type XXVIII, Sigma), and incubated overnight at 37°C. The lysate was extracted twice with phenol/chloroform (Section 4.3.3.1), then ethanol precipitated (Section 4.3.3.2).

Reagents:

Buffer C:	50mM Tris-HCl (pH 8.0), 10mM EDTA, 2% (w/v) N-lauryl sarkosine
Buffer D:	0.35M sorbitol, 50mM Tris-HCl (pH 8.0), 5mM EDTA, 0.1% (w/v) BSA, 1mM spermine and 1mM spermidine (both added from filter-sterilised stocks), 15mM β-mercaptoethanol (added just before use)
Buffer E:	0.35M sorbitol, 50mM Tris-HCl (pH 8.0), 10mM EDTA, 1mM spermine and 1mM spermidine (both added from filter-sterilised stocks)

4.3.5.2 Restriction digestion of plant DNA

Approximately 10μg of total genomic plant DNA extracted from either of the above extraction procedures (Sections 4.3.5.1.1 and 4.3.5.1.2) was digested in a total volume of 100-150μl with a 4-5 fold excess of restriction enzyme (Section 4.3.3.4) for 6-8 hours. The DNA was ethanol precipitated (Section 4.3.3.2) and resuspended in 15μl of TE. The DNA concentration and the completeness of digestion were checked by gel electrophoresis.

4.3.5.3 Preparation of gels for blots

5-10μg of DNA were loaded in each track. Gel electrophoresis conditions were as described in Section 4.3.3.5. Molecular weight markers (λ DNA digested with *Hin*dIII and *Eco*RI, as described in Section 4.3.3.5) were included but were removed prior to Southern blotting.

4.3.5.4 Southern blotting

(Based on the modification by Wahl *et al.* (1979) of the procedure developed by Southern (1975)). Following electrophoresis, the gel was placed in 0.25M HCl until the bromophenol blue marker dye had changed to a yellow colour (15-20 minutes). The gel was then rinsed in distilled water, placed in denaturing solution (1.5M NaCl, 0.5M NaOH) for 30 minutes at room temperature, with shaking, then transferred to neutralising solution (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.001M EDTA) for two 15 minute washes. DNA was then transferred onto 'Hybond-N'® nylon membrane (Amersham International, plc) by Southern transfer in 20× SSC for 5 hours. The membrane was rinsed carefully in 2× SSC then allowed to air-dry for 30 minutes, wrapped in 'Saran Wrap' (Dow Chemical Co.) and placed DNA side down on a U.V. transilluminator for 5 minutes to covalently bind the DNA to the filter.

4.3.5.5 Construction of a genomic library of Nicotiana tabacum

Outline of protocols: total genomic DNA was extracted from *Nicotiana tabacum* 'Xanthi' using the urea extraction method (Section 4.3.5.1.2) and purified by caesium chloride density gradient centrifugation (Section 4.3.5.1.3). The DNA was then digested to completion with *Hin*dIII (Section 4.3.5.2); fragments of 0.5-2.0kb were selected (Section 4.3.5.5.1) and ligated (Section 4.3.5.5.3) into a dephosphorylated pUC plasmid. The products of the ligation reaction were transformed into *E. coli* (Section 4.3.5.5.4) and bacteria containing recombinant plasmids were selected.

4.3.5.5.1 Size selection of DNA

a. using agarose gels: DNA of the required size was isolated following gel electrophoresis of restriction enzyme digested DNA by electro-elution onto DEAE paper, by the 'freeze-squeeze' method, by recovery from low melting point agarose or by eletroelution into a trough cut into the gel. Methods for the first three methods are given in Gaastra and Jørgensen (1984); the last technique is described by Ogden and Adams (1987).

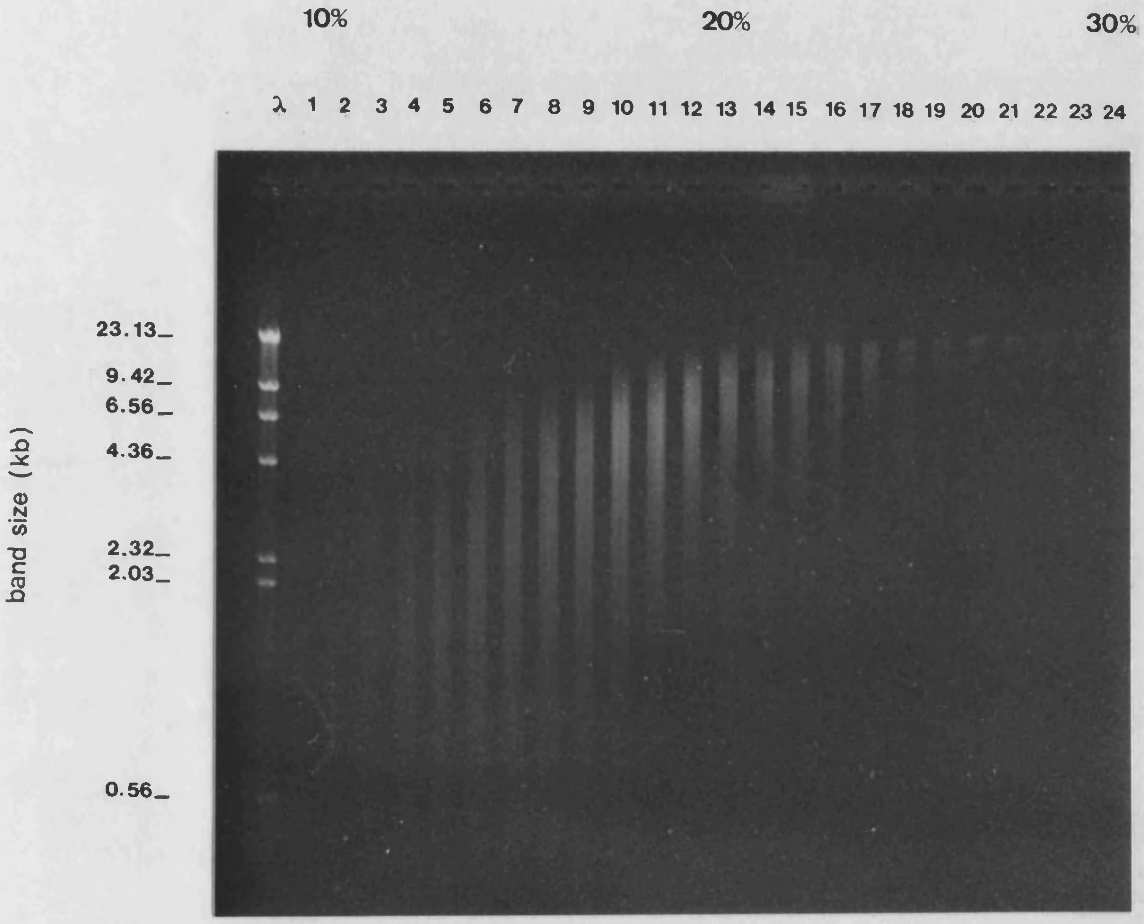
b. using a glycerol gradient: this is a modification of the method outlined in Chen *et al*, (1986). Five solutions were prepared containing 10% (v/v), 15%, 20%, 25% and 30% glycerol respectively in 10mM Tris-HCl (pH 8.0), 200mM NaCl and 2mM EDTA. A glycerol gradient was prepared in a 14ml MSE polycarbonate ultracentrifuge tube by carefully layering 2.7ml of each solution into the tube, with the glycerol concentration decreasing towards the top of the tube. 100μg of *Hin*dIII digested 'Xanthi' DNA was carefully layered onto the top of the gradient using a Pasteur pipette, then the gradient was centrifuged at 25000rpm for 16 hours at 4°C in a MSE Europa 75M ultracentrifuge. 500μl fractions were aliquoted into Eppendorf tubes; a 10μl sample from each fraction was run on a 0.7% (w/v) agarose gel to determine the size of the DNA in each fraction (Plate 12).

4.3.5.5.2 Cloning vectors

Sources: pUC13 and pUC18 were constructed by J. Messing and co-workers (Vieira and Messing, 1982; Yanisch-Perron *et al.*, 1985). *Escherichia coli* JM83 carrying the pUC18 plasmid was obtained from Bethesda Research Laboratories. The plasmid was extracted by alkaline lysis and purified by caesium chloride density gradient centrifugation (see below).

Plate 12: Size selection of tobacco genomic DNA

Hin dIII-digested DNA from *Nicotiana tabacum* , size-fractionated using a glycerol gradient. The percentage of glycerol in the gradient ranged from 10 to 30 as indicated.


10%
20%
30%
λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
band size (kb)
23.13
9.42
6.56
4.36
2.32
2.03
0.56

pUC13, digested by *Hin*dIII and dephosphorylated with bacterial alkaline phosphatase, was obtained from Pharmacia.

Structure: maps of pUC13 and pUC18 are shown in Figure 6. These plasmids both bear an ampicillin resistance gene and the *lac*Z gene, which confers the ability to break down the colourless BCIG (5,bromo-4,chloro-3,indolyl-β-D-galactopyranoside) into a characteristic blue compound. Within the *lac*Z gene is a synthetic polylinker containing several restriction sites (Figure 6).

Isolation of pUC18:

Plasmid extraction: 10ml of LB with ampicillin (Section 4.3.4.2) was inoculated with a single colony of JM83/pUC18 and was incubated, with vigorous shaking, overnight at 37°C. 100μl of this culture was used to inoculate 25ml of LB+Ap, which was incubated at 37°C until it had reached an optical density at 600nm of 0.6. This culture was mixed with 500ml fresh medium, and then incubated for a further 2.5 hours. Plasmid amplification was achieved by adding chloramphenicol (Section 4.3.4.2) to give a final concentration of 170μg/ml, then incubating the culture with vigorous shaking for 12-16 hours at 37°C.

Cells were harvested by centrifuging at 4000× *g* for 10 minutes at 4°C. The pellet was washed in 100ml ice-cold STE (0.1M NaCl, 10mM Tris-HCl (pH 7.8), 1mM EDTA), resuspended in 10ml solution I (50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA, with 5mg/ml lysozyme added just before use), then kept at room temperature for 5 minutes. 20ml fresh solution II (0.2N NaOH, 1% (w/v) SDS) was added and mixed in by inversion. The tube was kept on ice for 10 minutes. 15ml of ice-cold 5M potassium acetate (pH 4.8) was added, the solutions mixed by inversion, and the tube was returned to ice for 10 minutes. Chromosomal DNA and debris were removed by centrifugation at 20,000rpm for 20 minutes at 4°C; the supernatant was transferred to two 'Corex' tubes and the plasmid DNA was precipitated by adding 0.6 volumes of isopropanol. After 15 minutes at room temperature, the DNA was recovered by centrifugation at 12000 *g* for 30 minutes. The pellet was washed with 70% (v/v) ethanol, dried (Section 4.3.3.2) and resuspended in 4ml TE (pH 8.0).

Plasmid purification by CsCl density gradient centrifugation: exactly 1g of solid CsCl was added for each 1ml of plasmid solution. The solution was mixed gently until the salt had completely dissolved,

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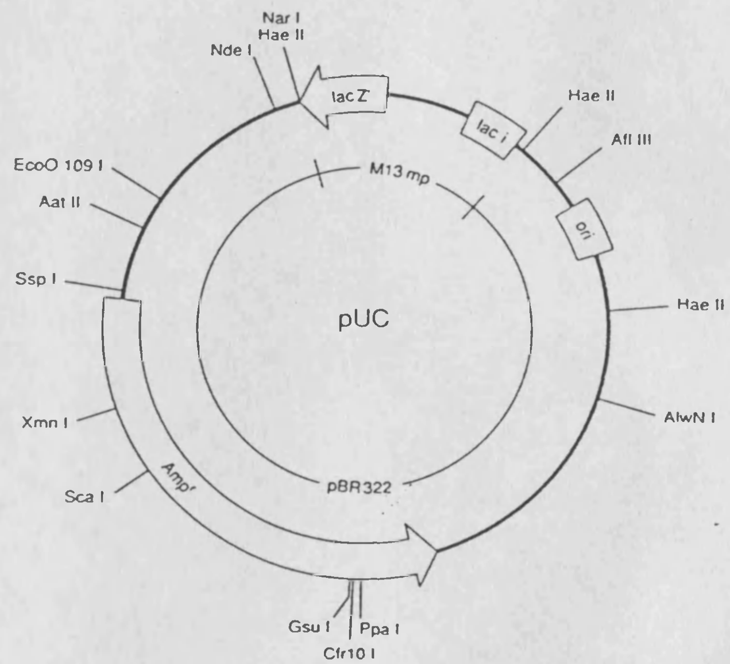
Figure 6: Plasmids pUC13 and pUC18

Above: The generalised structure of the pUC plasmids
Below: The multiple cloning sites (polylinkers) of pUC13 and pUC18



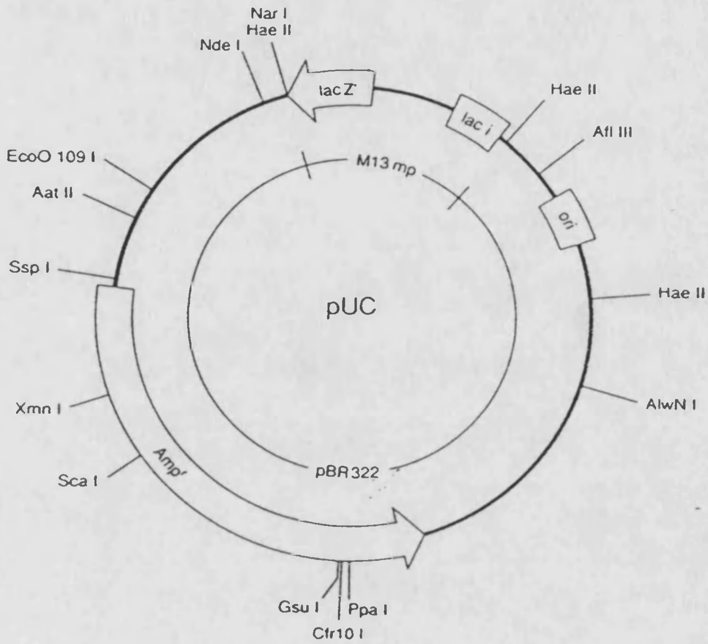
	1	2	3	4	(1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16)	5	6	7	8
pUC13	THR	MET	ILE	THR	pro	ser	leu	gly	cys	arg	ser	thr	leu	glu	asp	pro	arg	ala	ser	ser	ASN	SER	LEU	ALA
	ACC	ATG	ATT	ACG	CCA	AGC	TTG	GGC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GCG	AGC	TCG	AAT	TCA	CTG	GCC
						Hind III			*Pst* I		*Sal* I		*Xba* I		*BamH* I				*Sst* I		*EcoR* I			
											Acc I						*Xma* I							
											Hinc II						*Sma* I							

		1	2	3	4	5	6	(1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18)	7	8
pUC18		THR	MET	ILE	THR	ASN	SER	ser	ser	val	pro	gly	asp	pro	leu	glu	ser	thr	cys	arg	his	ala	ser	leu	ala	LEU	ALA
	ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC
						EcoR I		*Sst* I		*Kpn* I			*BamH* I		*Xba* I		*Sal* I		*Pst* I		*Sph* I		*Hind* III				
											Xma I						*Acc* I										
											Sma I						*Hinc* II										

facing page 84

Figure 6: Plasmids pUC13 and pUC18

Above: The generalised structure of the pUC plasmids
Below: The multiple cloning sites (polylinkers) of pUC13 and pUC18



1	2	3	4	(1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16)	5	6	7	8
THR	MET	ILE	THR	pro	ser	leu	gly	cys	arg	ser	thr	leu	glu	asp	pro	arg	ala	ser	ser	ASN	SER	LEU	ALA
ACC	ATG	ATT	ACG	CCA	AGC	TTG	GGC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GCG	AGC	TCG	AAT	TCA	CTG	GCC
					Hind III			*Pst* I		*Sal* I		*Xba* I		*Bam*H I				*Sst* I		*Eco*R I			
										Acc I						*Xma* I							
										Hinc II						*Sma* I							

	1	2	3	4	5	6	(1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18)	7	8
	THR	MET	ILE	THR	ASN	SER	ser	ser	val	pro	gly	asp	pro	leu	glu	ser	thr	cys	arg	his	ala	ser	leu	ala	LEU	ALA
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC
					*Eco*R I		*Sst* I		*Kpn* I			*Bam*H I		*Xba* I		*Sal* I		*Pst* I		*Sph* I		*Hind* III				
										Xma I						*Acc* I										
										Sma I						*Hinc* II										

then 0.8ml of 10mg/ml ethidium bromide was added for each 10ml of CsCl solution. The caesium chloride solution was transferred to a 14ml MSE polycarbonate ultracentrifuge tube, and was centrifuged in a MSE Europa 75M ultracentrifuge at 45000rpm for 36 hours at 20°C.

The lowest band on the gradient (closed circular plasmid) was removed with a hypodermic syringe. It was extracted repeatedly, with 1 volume of aqueous 5M NaCl, 10mM Tris-HCl (pH 8.5), 1mM EDTA, until no more pink colour was visible. Two volumes of H_2O and then 6 (original) volumes of ethanol were added to precipitate the DNA. After 1 hour at -20°C, the DNA was collected by centrifugation in a microfuge, washed with 70% (v/v) ethanol, dried (Section 4.3.3.2) and resuspended in 100µl TE (pH 7.5).

Dephosphorylation of vector DNA: linear pUC18 was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) according to the manufacturer's instructions: 250ng of *Hin* dIII-digested plasmid was incubated at 37°C with one unit of alkaline phosphatase (diluted from a stock solution with 50mM Tris-HCl (pH 8.0), 0.1mM EDTA) in CIP incubation buffer (50mM Tris-HCl, 0.1mM EDTA, pH 8.0) for 30 minutes. To stop the reaction, EDTA (pH 8.0) was added to a concentration of 50mM. The tube was then heated at 65°C for 45 minutes.

Since alkaline phosphatase cannot be completely inactivated by heat (Dale and Greenaway, 1984), the reaction was terminated by phenol extraction (Section 4.3.3.1) and ethanol precipitation (Section 4.3.3.2) after the recommended heat inactivation.

4.3.5.5.3 Ligation

The method was taken from King and Blakesley (1986). 100ng of linear, dephosphorylated plasmid and 20-40ng of size-selected tobacco genomic DNA fragments (*Hin*dIII digested) were incubated with 2.4 units of T4 DNA ligase (Northumbria Biologicals Ltd.) in ligation buffer† for 4 hours at 23-26°C. The total reaction volume was 20µl. The reaction was terminated by adding 1µl of 0.5M EDTA (pH 8.0) and the ligation products were diluted 5-fold prior to transformation.

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† 250mM Tris-HCl (pH 7.6), 50mM $MgCl_2$, 25% (w/v) PEG 8000, 5mM ATP and 5mM dithiothreitol

4.3.5.5.4 Transformation

The 'Standard Transformation Protocol' of Hanahan (1985) was used. Several colonies of *E. coli* TB-1 from a freshly streaked SOB plate (Section 4.3.4.2) were dispersed in 1ml SOB by vortexing; these cells were then inoculated into 50ml SOB in a 500ml Erlenmeyer flask. The culture was incubated at 37°C with moderate agitation until it reached a viable cell density of 4-7× 10^7 viable cells/ ml. This corresponds to an optical density at 550nm of 0.45-0.55 units. The culture was chilled on ice in two 50ml polypropylene centrifuge tubes, for 15 minutes, then the cells were pelletted by centrifugation at 1000× *g*. The supernatant was drained off thoroughly, and the cells were resuspended in one third of the original culture volume of TFB (see below) and kept on ice for 15 minutes. The cells were pelletted again and resuspended in 1/12.5 of the original culture volume of TFB. 7μl of DnD (see below) was added for each 200μl of the cell suspension, and was mixed by swirling. The tube was kept on ice for 10 minutes, then another 7μl of DnD was added for each 200μl cell suspension. The cells were kept on ice for a further 20 minutes.

The diluted products of the ligation reaction (Section 4.3.5.5.3) were added to 200μl aliquots of competent cells in chilled, thin-walled 'Pyrex' tubes. After 30 minutes on ice, the cells were given a 42°C heat shock for 90 seconds, then cooled on ice for 2 minutes. 800μl of SOC (see below) was added to each tube, and the cells were incubated at 37°C for 60 minutes, with moderate agitation. The cells were spread onto LB+Ap plates with 40μg/ml BCIG (see below). The plates were incubated, inverted at 37°C overnight.

Notes: All glassware was acid-washed and rinsed in several changes of distilled water. Where possible, plasticware was rinsed well to remove any surfactants used in the manufacturing process.

Alternative transformation protocols:

i. *E. coli* JM109 was used instead of TB-1 to make competent cells. This has a defective *lac*Z gene (Section 4.3.4.1) and so the inducer IPTG (isopropyl β-D-thiogalacto-pyranoside) must be added with the BCIG in order to get a blue colour. A filter-sterilised IPTG stock solution at 25mg/ml in H_2O was stored at -20°C. The working concentration of the IPTG was 40μg/ml.

ii. To reduce the quantities of IPTG and BCIG required, transformed cells can be plated in a thin layer of top agar containing IPTG and BCIG on top of ordinary LB plates.

iii. For convenience, frozen competent cells were also prepared, using the 'Frozen Storage Protocol 2' of Hanahan (1985). This differs from the protocol above in two ways: firstly, it uses FSB, which is the same as TFB but with 10% (w/v) redistilled glycerol, and secondly, 100% (w/v) DMSO is used in place of DnD. Cells were frozen in 210µl aliquots and stored at -70°C.

Control reactions: several controls were routinely done to monitor the steps of ligation and transformation:

i. To check the background level of antibiotic resistance, non-transformed competent cells were plated out onto LB+Ap.
ii. The success of the alkaline phosphatase treatment was assessed by preparing a ligation reaction without insert DNA.
iii. Transformation efficiency was measured by finding the number of transformed cells obtained from a known amount (10pg-1ng) of intact plasmid DNA.

Transformation reagents:

TFB:	100mM KCl (ultrapure), 45mM $MnCl.4H_20$, 10mM $CaCl_20.2H_20$, 3mM hexamine cobalt (III) chloride (Aldrich), 10mM K-MES (added from a 0.5M stock solution— see below). TFB was filter-sterilised through a pre-rinsed 0.2µm filter into sterile bottles and was stored at 4°C.
0.5M K-MES:	an 0.5M solution of MES (2[N-morpholino]ethone sulphonic acid) was Adjusted to pH 6.3 using concentrated KOH. It was filter-sterilised and stored at -20°C.
DnD:	1M dithiothreitol, 90% (v/v) dimethylsulphoxide (Fluka), 10mM potassium acetate (added from a sterile 1M stock, pH 7.5). This solution was not sterilised; it was stored at -20°C.
SOC:	identical to SOB (Section 4.3.4.2) except for the addition of 20mM glucose, added from a filter-sterilised 2M stock solution.

BCIG: (5,bromo-4,chloro-3,indolyl-β-D-galactopyranoside). A filter-sterilised stock solution at 25mg/ml in dimethylformamide was stored at -20°C.

4.3.5.5 Storage and analysis of clones

Storage: clones were stored on 'Hybond-N' membranes on agar plates, as described in Section 4.3.4.4. Glycerol stocks of individual clones were also made, as described in Section 4.3.4.4.

Analysis of copy number:

Colony hybridisation: this protocol was based on that in 'Membrane transfer and detection methods' (1985). A grid of clones on a 'Hybond-N' membrane was prepared as described in Section 4.3.4.4. Control strains with plasmids but no inserts were also included. The membrane was placed on a LB+Ap agar plate and was incubated at 37°C until the colonies were 1mm in diameter. The membrane was placed, colony side up, on top of a series of wads of Whatman 3MM paper soaked in the following solutions:

i. 10% (w/v) SDS (3 minutes)
ii. 1.5M NaCl, 0.5M NaOH (7 minutes)
iii. 1.5M NaCl, 0.5M Tris-HCl (pH 7.2), 0.001M EDTA (3 minutes)
iv. as for (iii)

The membrane was rinsed in 2× SSC, allowed to air-dry, and was wrapped in 'Saran Wrap' (Dow Chemical Co.) and placed, colony side down, on a u.v. transilluminator for 4 minutes to bind the DNA onto the membrane.

The membrane was hybridised with oligo-labelled, sheared total genomic *Nicotiana tabacum* 'Xanthi' DNA, as described in Section 4.3.5.6, and autoradiographed using pre-flashed film (Section 4.3.5.6.6).

Dot blotting: (protocol from Mason and WIlliams (1985)). A Schleicher and Schull vacuum-filtration manifold system was set up as follows: a piece of filter paper (supplied with the system) was wetted with 2× SSC, placed on the base of the manifold and covered with a piece of 'Hybond-N' membrane cut to size. The top section of the manifold was replaced, then gentle suction was applied using a water pump, so that 500µl of 20× SSC took at least 5 minutes to pass through the filter.

Plasmid was extracted from each clone and from JM83/pUC18 by alkaline lysis mini-preparations (see below) and resuspended in 50µl 1mM EDTA, 20mM Tris-HCl (pH 7.6). The samples were heated in a boiling water bath for 10 minutes, then mixed with an equal volume of 1M NaOH and incubated at room temperature for 20 minutes. The samples were put on ice, then 400µl of neutralisation buffer (1.5M NaCl, 1M Tris-HCl (pH 7.5)) was added to each tube and the samples were pipetted into the wells of the manifold. When filtration was complete, the membrane was placed on a piece of Whatman 3MM paper, allowed to air-dry, wrapped in 'Saran Wrap' and placed, DNA side down, on a u.v. transilluminator for 4 minutes. The membrane was hybridised with sheared, total genomic DNA from a seed-grown *Nicotiana tabacum* 'Xanthi' plant, and was autoradiographed using pre-flashed film (Section 4.3.5.6.6).

Determination of insert size : plasmid minipreparations were carried out according to the method of Birnboim and Doly (1979) described in Maniatis *et al.* (1982): Each clone was inoculated into 5ml of liquid LB+Ap in a glass universal tube; the cultures were incubated at 37°C overnight, with vigorous shaking. The cells from 1.5ml of the overnight culture were collected by centrifugation for 1 minute in a microfuge, the supernatant was removed by aspiration, and the pellet resuspended by vortexing in 100µl ice-cold 50mM glucose, 10mM EDTA, 25mM Tris-HCl (pH 8.0). The samples were kept at room temperature for 5 minutes, then 200µl freshly prepared, ice-cold 0.2N NaOH, 1% (w/v) SDS was added. The tubes were inverted rapidly several times to mix the contents. Chromosomal DNA and proteins were then precipitated by the addition of 150µl ice-cold potassium acetate (pH 4.8); the tubes were vortexed, inverted, for 10 seconds and kept on ice for 5 minutes. The precipitate was pelletted by centrifuging for 5 minutes at 4°C, and 400µl of the supernatant was carefully transferred to a fresh Eppendorf tube, extracted with phenol/chloroform (Section 4.3.3.1) and ethanol precipitated (Section 4.3.3.2). The pellet was resuspended in 50µl TE (pH 8.0) containing 20µg/ml DNase-free RNase (Section 4.3.2). 5µl of the plasmid preparation was digested with 1 unit of *Hin*dIII for 2 hours at 37°C (Section 4.3.3.4). The sizes of the inserts were determined by running the digested plasmid samples on an agarose gel (Section 4.3.3.5) with λ *Hin*dIII markers.

4.3.5.6 Methods for radioactive labelling and hybridisation

4.3.5.6.1 Preparation of sequences to be labelled

Plasmids containing the desired insert were purified as described in Section 4.3.5.4.5 and digested with *Hin*dIII to cut out the insert. A known amount of the digested DNA was loaded onto a 0.6% (w/v) low melting point agarose gel (Sigma agarose, type VII), and the insert was separated from the plasmid by gel electrophoresis. A gel slice containing the insert band was cut out and weighed, allowing calculation of the DNA concentration. Just prior to labelling, the agarose was melted and the DNA denatured by heating in a boiling water bath for 7 minutes.

4.3.5.6.2 Oligolabelling

This protocol was developed by Feinberg and Vogelstein (1984). The reaction was set up in a screw-capped Eppendorf tube as follows :

H_2O	to a final volume of 15μl
OLB[a]	3μl
BSA[b]	0.6μl
DNA	10ng
dCTP[c]	1.5μl
Klenow[d]	0.6μl

Notes:

a. Oligolabelling buffer (OLB) was made by mixing the following solutions in the ratio of 2 : 5 : 3 by volume. It was stored at -20°C.

Solution A:	625μl 2M Tris-HCl (pH 8.0), 25μl 5M $MgCl_2$, 350μl H_2O, 18μl β-mercaptoethanol, 5μl each of dATP, dTTP and dGTP (each triphosphate was dissolved in 3mM Tris-HCl (pH 7.0), 0.2mM EDTA at 0.1M)
Solution B:	2M HEPES titrated to pH 6.6 with NaOH
Solution C:	hexadeoxyribonucleotides (Pharmacia cat. no. 27-2166-01) evenly suspended in 3mM Tris-HCl (pH 7.0), 0.2mM EDTA at 90 OD

units/ml.

b. 10mg/ml enzyme grade BSA (bovine serum albumin) from Boehringer Mannheim.

c. Deoxycytidine 5'-[α-^{32}P] triphosphate (triethylammonium salt) in stabilised aqueous solution at 10μCi/μl (from Amersham International, plc).

d. 1 unit/μl from Northumbria Biologicals Ltd.

The reaction was allowed to proceed for at least 5 hours and was then terminated by the addition of 85μl stop solution (20mM NaCl, 20mM Tris-HCl (pH 7.5), 2mM EDTA, 0.25% (w/v) SDS). The probe was denatured by boiling for 5 minutes before hybridisation (Section 4.3.5.6).

Measurement of incorporation: 1μl of the terminated oligolabelling reaction was diluted with 9μl water and spotted onto the centre of a Whatman GF/C glass fibre disc.

A second 1μl was added to 100μl of salmon sperm DNA (500μg/ml in 20mM EDTA). The DNA was precipitated by adding 5ml ice-cold 10% (w/v) TCA (trichloroacetic acid) and chilling on ice for 15 minutes; it was collected by filtering through a second GF/C disc. The disc was washed 6 times with 5ml ice-cold TCA and then with 95% ethanol.

Both filters were dried under a heat lamp, and the Cerenkov radiation from each was measured (without scintillation fluid) using a scintillation counter. The first disc gives the total counts in the sample, while the second gives a measure of the radioactivity incorporated into nucleic acids.

4.3.5.6.3 Pre-hybridisation

Filters were pre-wetted in 1.5× SSPE and placed in a 100ml polypropylene measuring cylinder with 5ml pre-hybridisation solution (1.5× SSPE, 0.5% (w/v) 'Marvel'® dried milk powder (Cadbury), 1% (w/v) SDS and 6% (w/v) PEG 8000) and 20μg/ml denatured salmon sperm DNA (see below). The cylinder was sealed with a rubber bung pierced with a syringe needle, and pre-hybridisation was carried out for at least 4 hours at 65°C in a rotisserie oven (Bachofer, West Germany). Sheared salmon sperm DNA (1mg/ml) was denatured just prior to addition to the pre-hybridisation solution by heating in a boiling water bath for 5 minutes.

4.3.5.6.4 Hybridisation

The pre-hybridisation solution was replaced with 5ml fresh, pre-warmed solution, then the oligo-labelled, denatured probe (Section 4.3.5.5.2) and 20µg/ml denatured salmon sperm DNA (see above) were added. Hybridisation was at 65°C for at least 12 hours.

4.3.5.6.5 Washes

After hybridisation, the filters were washed at 65°C with 2× SSC (two 15 minute washes), then in 2× SSC with 0.1% (w/v) SDS (30 minutes), followed by a 10 minute high stringency wash in 0.1× SSC. The filters were then allowed to air-dry for up to 30 minutes, and were wrapped in 'Saran Wrap'.

4.3.5.6.6 Autoradiography

Autoradiography was at -80°C using Fuji RX medical X-ray film in autoradiography cassettes (Genetic Research Instrumentation Ltd.) with Dupont 'Cronex® Lightning Plus' intensifying screens. Where stated, autoradiography film was pre-flashed (Laskey, 1984) prior to use; this is necessary to make the response of the film proportional to the amount of radioactivity on the membrane. Autoradiographs were developed by immersion for 5 minutes in Kodak D19 developer, then rinsed in water, fixed for 5 minutes in Kodak 'Unifix' fixer (dilution B, i.e. 260g/l), then rinsed thoroughly in tap water.

4.3.5.6.7 Removal of probes and re-use of blots

Filters were deprobed by incubating at 45°C in 0.4M NaOH for 30 minutes, then at 45°C in 0.1× SSC, 0.1% (w/v) SDS, 0.2M Tris-HCl (pH 7.5) for 30 minutes. The deprobed filters were autoradiographed to check that probe removal was complete. Filters were successfully probed and reprobed up to 5 times.

4.4 Experimental work, results and discussion

Many technical problems were encountered during the cloning and RFLP analysis of *Nicotiana tabacum* DNA. Some factors of particular importance to the success of these techniques are discussed below.

4.4.1 Comments on techniques

4.4.1.1 DNA extraction

Tobacco DNA isolated using the urea extraction method (Section 4.3.5.1.2) was always very viscous, making it reluctant to digest completely. An increased volume of extraction buffer and repeated phenol/chloroform extractions did not improve the quality of the DNA. Growing plants in the dark for 3-4 days prior to DNA isolation did reduce the viscosity, suggesting that high starch levels may be part of the problem. With these modifications, 'clonable' DNA was produced, but DNA isolated using this method could rarely be digested completely, and so the CTAB extraction method (Section 4.3.5.1.3) was preferred for the preparation of DNA for Southern blots. CTAB binds to nucleic acids, allowing them to be precipitated while leaving the majority of polysaccharides in solution (Murray and Thompson, 1980).

4.4.1.2 Restriction digestion

Even with DNA prepared using the CTAB method, complete digestion was not possible with all restriction enzymes tested. Enzymes were graded according to their ability to digest DNA, as judged by gel electrophoresis (Table 15). Part of the reason for the resistance to digestion of plant DNA in general is thought to be the high level of methylation of bases that occurs in plants: many cytosine residues in plants are methylated, especially those in $^{5'}$ CG $^{3'}$ and $^{5'}$ CXG$^{3'}$ sequences (Gruenbaum *et al.*, 1981) and many restriction enzymes cannot cleave if there are methylated bases in their recognition site (Nelson and McClelland, 1989). It can be seen that those enzymes having recognition sites containing $^{5'}$ CXG $^{3'}$ sequences, such as *Pst*I and *Hpa*II, do not reliably digest tobacco DNA; this confirms the results of Gruenbaum *et al.* (1981). Not all enzymes with recognition sites containing $^{5'}$ CG $^{3'}$ sequences fail to cut plant DNA — on the contrary, *Taq*I was the best enzyme tested. This enzyme has been shown to be insensitive to cytosine methylation (Nelson and McClelland, 1989). Some other enzymes that are insensitive to methylation, such as *Kpn*I, though, are poor at digesting

TABLE 15. Ability of restriction enzymes to digest tobacco DNA

Restriction enzymes were graded according to their ability to digest to completion the DNA of *Nicotiana tabacum*. These abilities are compared with the recognition sites below.

EXTENT OF DIGESTION	RESTRICTION ENZYME	RECOGNITION SITE 5' 3'
Excellent	*Taq* I	TCGA
Very good	*Dra* I	TTTAAA
	Mnl I	CCTC
	Rsa I	GTAC
Good	*Alu* I	AGCT
	Hin dIII	AAGCTT
	Sau 3AI	GATC
Fair	*Bam* HI	GGATCC
	Bgl II	AGATCT
	Eco RI	GAATTC
	Msp I	CCGG
Poor	*Bsc* I	ATCGAT
	Bst UI	CGCG
	Hpa II	CCGG
	Kpn I	GGTACC
	Pst I	CTGCAG
	Sma I	CCCGGG

tobacco DNA, and so methylation cannot be the only factor influencing digestibility of DNA. It is possible that some enzymes are more sensitive than others to the presence of impurities in the digestion reaction.

The reliability of *Taq*I may be due to the high temperature of the reaction, which was 65°C. The improved digestion could be a result of the reduced viscosity of the DNA at this temperature, rather than to a permanent alteration, since heating of other samples to 65°C prior to digestion at 37°C did not improve digestion. This suggests that in order to optimise digestions, the purity of the DNA would have to be improved. Although caesium chloride density gradient ultracentrifugation could have been used to further purify DNA samples, it was considered inappropriate for this project due to the impracticality of using it for a large number of samples.

In general, restriction digestion conditions were optimised by using large reaction volumes, so that impurities were diluted, and an excess of restriction enzyme. Those enzymes that were found to be unreliable were avoided.

4.4.1.3 Preparation of Southern blots

In order to detect a particular sequence on a Southern blot, a certain amount of total genomic DNA must be present on the blot. This amount depends on the total size of the genome, and on the number of copies of that sequence present in the genome. Bernatzky and Tanksley (1986a) used 3μg of DNA per lane to detect single copy sequences in tomato, which has a genome size (2C value) of 1.4pg. Tobacco has a much larger genome, with estimates ranging from 6.6pg per cell (Goldberg *et al.*, 1978) to 11.71pg per cell (Narayan, 1987). Initially, therefore, 5μg of DNA was loaded per lane, but not all single copy probes showed detectable hybridisation to these blots, and so the amount loaded was increased to 7-8μg per track. When using such large amounts of DNA, the concentration of the DNA (and also of any impurities in the DNA samples) is unavoidably high. This results in a high sample viscosity, which may have been the cause of the marked U-shape of many bands; it is suggested that the high surface tension created at the corners of the wells impedes the migration of the DNA†. This problem was not resolved, but could have been reduced by using a gel electrophoresis system that

† Scott (1988) suggests that U-shaped bands occur when the DNA sample is incompletely re-dissolved.

allowed larger volumes to be loaded per well, so that sample concentration could be reduced. Any factors that increased the efficiency of Southern transfer, oligo-labelling, hybridisation and autoradiography would reduce the minimum amount of DNA that needed to be loaded, thus also improving the situation.

The large quantity of DNA loaded means that even very slight differences in sample concentrations are exaggerated greatly. Since the DNA concentration can affect DNA mobility and band shape, as discussed above, this can lead to problems in interpreting autoradiographs. It is therefore important that DNA concentration is accurately determined. Spectrophotometric estimation of DNA concentration was found to be inaccurate, and often misleading, for DNA not subjected to caesium chloride density gradient purification, and although gel electrophoresis was a much more reliable method, it still did not give the precise determination of concentration necessary to ensure equal loading. The problem will only be resolved by the development of DNA extraction methods that produce DNA of high enough purity for accurate spectrophotometric analysis. One point of interest is that uneven loading between tracks was more of a problem with blots of somaclone DNA than with those of control plant DNA: it is suggested that the more variable yields and viscosities of somaclone DNA were due to physiological and/or biochemical differences between regenerated plants.

4.4.1.4 Oligolabelling

Especially when using single copy probes, this detection system is close to the threshold of its resolution, and so it is crucial that the most important factors affecting resolution are optimised. The specific activity of the α-^{32}P-dCTP is critical: the higher the specific activity, the better. Freshly made oligolabelling buffer was also found to give much higher incorporations than buffer that had been frozen and thawed a few times.

4.4.1.5 Autoradiography

Sensitivity of autoradiography film was also an important factor, and was found to vary considerably between brands. Kodak AX film was very insensitive; Kodak CX was better but not reliable for the detection of single copy sequences; Fuji RX was the most sensitive of the three types used.

4.4.1.6 Transformation: problems and solutions

A number of problems were encountered during the production of a tobacco genomic library. These problems, their likely causes, and remedies are discussed below.

1. Some batches of ampicillin were ineffective, allowing lawns of ampicillin-sensitive, non-transformed cells to grow on LB+Ap plates. This was thought to be due to the variation in the amount of NaOH used to dissolve the antibiotic. No problems were found when the sodium salt of ampicillin was used; this is readily soluble in water.

2. Background white colonies of JM109 grew on plates without DNA and/or white colonies without plasmids were found on transformation plates. Tests showed that JM109 stocks did contain a low number of ampicillin resistant cells. This problem was not encountered with TB-1. Non-transformed cells *could* grow on ampicillin media when fairly close to ampicillin resistant transformants, due to the diffusion of β-lactamase, so it was important to screen for blue- or white-ness as soon as possible, before satellite colonies and "false clones" appeared.

3. The blue/white colour distinction was poor when colonies were buried in top agar—a better colour change was seen when cells were plated directly onto the surface of BCIG plates. The blue colour developed more rapidly in TB-1 than in JM109, possibly because the latter requires the inducer IPTG in order to respond. Even with TB-1, though, it was often necessary to plate out putative clones a second time onto fresh LB+Ap with BCIG to get a distinct colour change.

4. As emphasised by Hanahan (1985), high grade reagents and scrupulously cleaned equipment were vital requirements for high transformation efficiencies. For example, Difco yeast extract gave better transformation efficiencies than Oxoid yeast extract, and it was important to use highly pure water. A transformation efficiency of 5×10^{6}- 5×10^{7} transformants/μg pUC18 was routinely obtained.

5. The background level of blue colonies i.e. those containing plasmids without inserts, was often high. This could have been due to ineffective dephosphorylation. However, according to the PNP test (Dale and Greenaway, 1984), the alkaline phosphatase used was active. The same symptoms could have been due to incomplete restriction digestion of the vector DNA, so that some remained intact and was never dephosphorylated. On an agarose gel, the vector appeared

to be completely in a linear state, but even a trace level of intact plasmid could have given a significant background level of blue colonies.

6. Despite routinely high transformation efficiencies, it was frequently the case that no clones were obtained. The ligation system itself was tested by religating *Hin*dIII digested λ DNA and checking it on a gel, and by comparing the number of transformants obtained with (i) cut vector and (ii) cut, religated vector. Both methods showed that the ligation reaction itself worked. It was found that dephosphorylation did reduce the ability of the vector to ligate to insert DNA; this, together with problems encountered in cutting out inserts later, suggests that dephosphorylation can damage the vector. This has been reported by Kuziel and Tucker (1987) in λ cloning. More clones (but also, of course, many more non-recombinant transformants) were obtained when non- dephosphorylated vector was used. Pure λ DNA fragments were cloned successfully, as were fragments of *Arabidopsis* DNA size-selected using DEAE paper (Section 4.3.5.5.1) (results not shown), but tobacco genomic DNA was always recalcitrant, suggesting that impurities in the DNA inhibited ligation. Tobacco DNA that had been size-selected by any method that used agarose gels (see Section 4.3.5.5.1) was not amenable to cloning, whereas many clones were obtained from DNA size-selected on glycerol gradients. This latter point was found to be the most important factor for successful cloning of tobacco genomic DNA.

4.4.2 ANALYSIS OF CLONES

4.4.2.1 Estimation of copy number of cloned sequences

The copy numbers of some of the 142 cloned sequences obtained was estimated by probing colony blots and dot blots (Section 4.3.5.4.5) with total genomic DNA. The results are shown in Plates 13 and 14. The sequences were divided into 3 categories by the strength of their hybridisation—highly repeated, medium copy number and low or single copy number. The results are shown in Table 16.

Firstly it can be seen that there is a discrepancy between the data from the two different methods. Although both agree regarding the proportion of highly repeated sequences, colony hybridisation gave a higher figure for low copy number sequences than did dot blotting. Both Landry and Michelmore (1985) and Figdore *et al.* (1988) found that colony hybridisation failed to detect some repeated sequences. The unreliability of this method may be due to the variability in the growth rate of different colonies on the filter. Dot blotting may not be absolutely reliable (Figdore *et al.* (1988)) but this estimate is probably closer to the actual proportions of different sequences. It is close to the figure of 70% repeated sequences derived by Goldberg *et al.* (1978) from the reassociation kinetics of *Nicotiana tabacum* DNA. In both this experiment and my work, the level of repetitivity may be overestimated due to the presence of two related genomes in this amphidiploid plant.

My clones were made from total genomic DNA from leaves. and therefore a significant number of them will represent chloroplast DNA. To identify those clones that were of chloroplast origin, chloroplast DNA was isolated (Section 4.3.5.1.4) and was used as a probe on the de-probed colony blot. The result is shown in Plate 13B.

Comparison of the results of colony hybridisation using the two different probes (Plate 13A and B) shows that there are some differences. It is difficult to compare the intensities of hybridisation for low copy number sequences, but of 20 high copy number sequences on the total genomic blot, 5 (25%) showed more hybridisation with the chloroplast DNA probe, 3 (15%) showed about the same degree of hybridisation while the remainder (60%) showed less hybridisation. This suggests that up to 25-40% of the cloned sequences could be of chloroplast DNA or of sequences present in both the nucleus and the chloroplast. The background hybridisation of the chloroplast DNA probe to all of the clones (Plate 13B) may have been due to contamination of the probe with nuclear DNA: restriction

Plate 13: Colony hybridisation

Hybridisation of (A) total genomic DNA and (B) chloroplast DNA
to clones of *Nicotiana tabacum* DNA in *E. coli* TB-1.
Colonies marked C are controls containing plasmids without inserts.
Clones used in subsequent experiments are indicated.

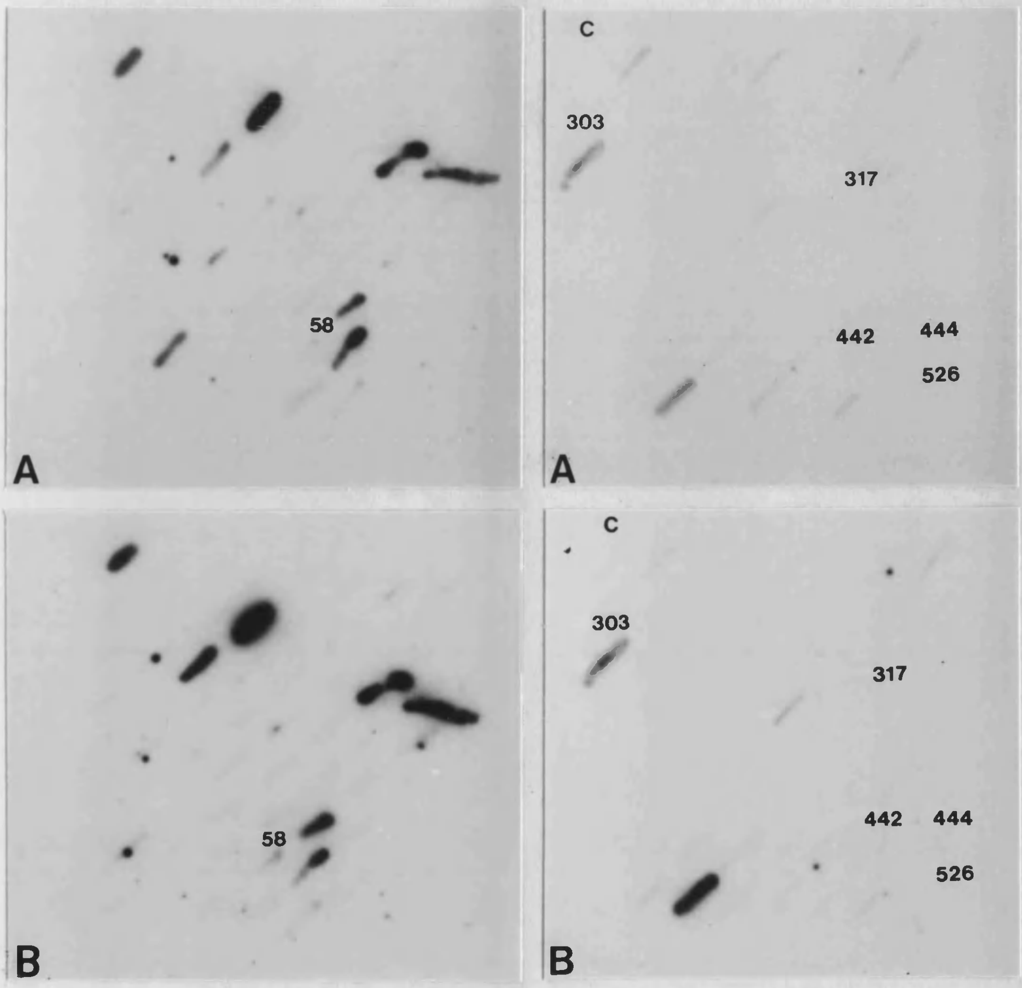

58
A
C
303
317
442
444
526
A
58
B
C
303
317
442
444
526
B

Plate 14: Dot-blotting

Hybridisation of total genomic DNA to dot-blotted plasmid mini-preparations of known clones and of a control with plasmid but without insert (C). Clones used in subsequent experiments are indicated.

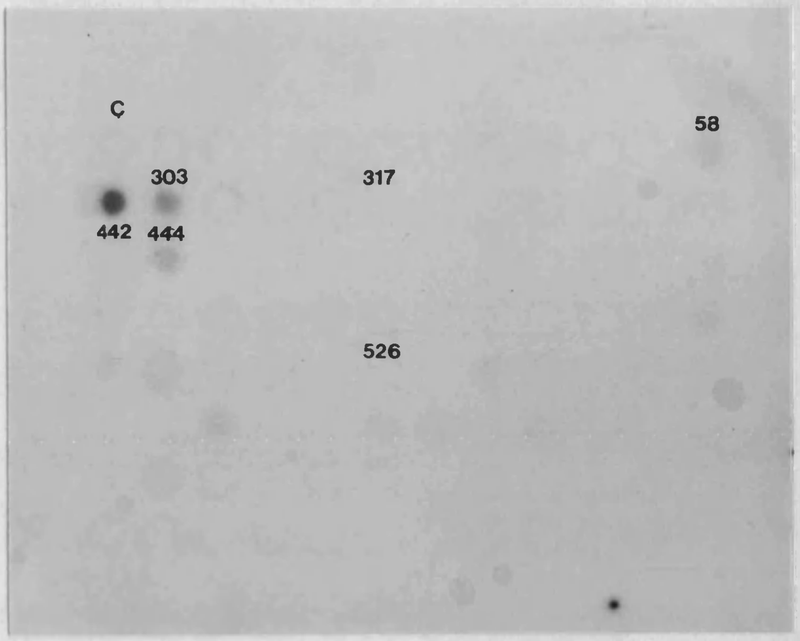

C
58
303
317
442
444
526

TABLE 16. Copy numbers of genomic clones

The copy numbers of cloned sequences were determined in two ways, by colony hybridisation and by dot blotting; the sequences were divided into three categories based on the strength of their hybridisation to total genomic DNA

SEQUENCE CLASS	TECHNIQUE	
	COLONY HYBRIDISATION	DOT BLOTTING
Highly repeated	11.4%	12.2%
Medium copy number	46.8%	63.4%
Low or single copy	41.8%	24.4%

TABLE 17. Size of inserts in recombinant plasmids

Insert size was determined by plasmid mini-preparations by alkaline lysis, followed by restriction digestion to release the plasmid. Different fractions from a glycerol gradient (see Section 5.3.5.5.1) gave different ranges of insert size:

FRACTION NO.	APPROXIMATE % GLYCEROL (v/v)	RANGE OF INSERT SIZES (kb)
10	17	0.5-2.2
11	18	0.7-3.0
20	26	0.6-5.0

digestion of the "chloroplast" DNA used as a probe produced clear bands on an agarose gel, but with a background smear suggestive of some nuclear DNA contamination.

4.4.2.2 Determination of insert size

Insert size was determined using the procedure described in Section 4.3.5.4.5. The results are shown in Table 17. If these data are compared with the photograph of the size-fractionated DNA (Plate 12), it will be noticed that the maximum fragment size in each size fraction appears to increase, whilst the lower limit of insert size remains constant. This is probably because although the majority of DNA fragments in a fraction are in a fairly limited range, there will also be smaller fragments which are not visible on a gel. These smaller fragments will be cloned more easily than larger fragments.

4.4.3 Detection of genetic variation using RFLPs

In order to examine the potential use of RFLP analysis in the measurement of genetic variation, different restriction enzyme/probe combinations were screened for their ability to detect variation between different species, cultivars and individual seed-grown and tissue culture-derived plants. Finally, an investigation was made into the extent of variation within an individual plant.

4.4.3.1 Comparison of different Nicotiana species and 3 other Solanaceous species

Total genomic DNA was isolated from potato (*Solanum tuberosum* c.v. Desirée), tomato (*Lycopersicon esculentum* c.v. Alicante), pepper (*Capsicum annuum* c.v. Early Cal Wonder), *Nicotiana tabacum* c.v. Xanthi and c.v. White Burley, *N. sylvestris*, *N. megalosiphon*, *N. glutinosa*, *N.debneyi* and *N. rustica* as described in Section 4.3.5.1. The DNA was digested with *Eco*RI (Section 4.3.5.2), and 5µg of cut DNA was run on a 0.8% agarose gel and transferred to 'Hybond-N'® by Southern blotting (Section 4.3.5.4). The filter was probed with a 2.6kb, low copy number 'Xanthi' genomic clone (#317) and autoradiographed (Section 4.3.5.6). The results are shown in Plate 15 and in diagrammatic form in Figure 7.

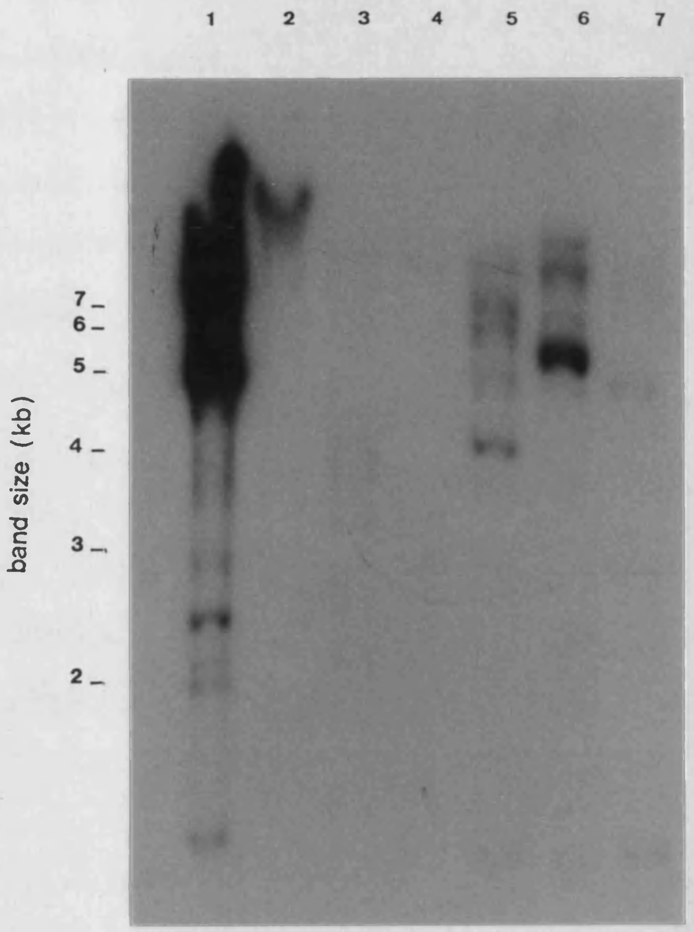
At this stringency (i.e. high), there was no detectable hybridisation of the probe to potato, tomato, pepper or *N. glutinosa* DNA. Only highly homologous sequences would be able to hybridise at this stringency; the lack of hybridisation of the species other then *Nicotiana* is therefore likely to be a reflection of the evolutionary divergence between these species. Similar conclusions were made by

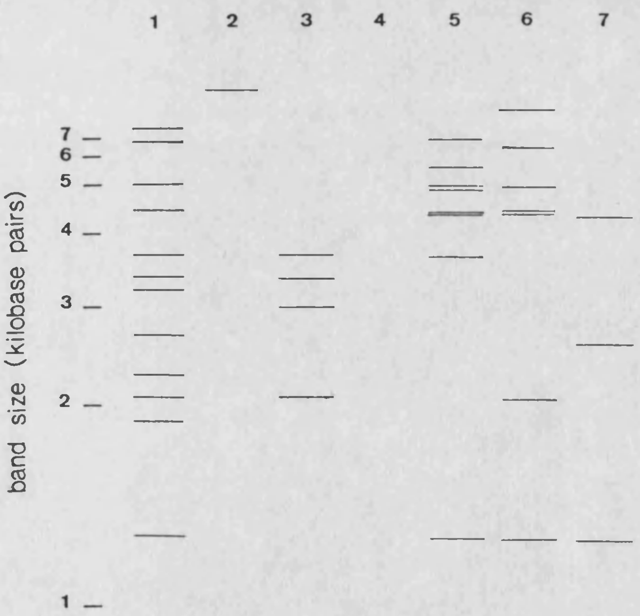
Plate 15: Detection of RFLPs between *Nicotiana* species

Eco RI digestion of the DNA of 7 *Nicotiana* species and three other members of Solanaceae, probed with a 2.6kb low copy number 'Xanthi' genomic clone (#317). *Track contents:* (1) *Nicotiana tabacum* c.v. 'Xanthi', (2) *N. sylvestris* , (3) *N. megalosiphon* , (4) *N. glutinosa* , (5) *N. debneyi* , (6) *N. tabacum* c.v. 'White Burley', (7) *N. rustica* .

Figure 7:

Diagrammatic representation of the autoradiograph above, compiled from several different exposures.


1
2
3
4
5
6
7
band size (kb)
7
6
5
4
3
2


1
2
3
4
5
6
7
band size (kilobase pairs)
7
6
5
4
3
2
1

Zamir and Tanksley (1988) who found that no highly repeated sequences, and only 40% of lower copy number tomato clones, hybridised to tobacco DNA. In order to assess the degree of relatedness between these species, it would be necessary to reduce the stringency of the hybridisation conditions. The failure of the probe to hybridise to *Nicotiana glutinosa* DNA is surprising, though, especially as this species is in the same subgenus as *Nicotiana tabacum* (Goodspeed, 1954). There was slightly less DNA in this track on the gel, but the difference does not seem sufficient to explain the complete lack of hybridisation.

It is evident from Figure 7 that this probe revealed RFLPs between the other *Nicotiana* species: each species had a unique band pattern. This shows that it would be feasible to use RFLPs for species identification in the genus *Nicotiana*. In order to assess the possible value of RFLPs in measuring relatedness, the number of bands shared between each possible pair of species was calculated from Figure 7. The results are shown in Table 18. The most similar pattern to 'Xanthi' was the other *Nicotiana tabacum* cultivar, 'White Burley'. From these data, the order of relatedness of the other species to *N. tabacum* 'Xanthi' appears to be *N. debneyi* (closest), *N. megalosiphon*, *N. rustica* and then *N. sylvestris* (most distant). It is surprising that there are not more bands shared by *N. tabacum* and *N. sylvestris*, since *N. sylvestris* is supposed to be one of the parents of the amphidiploid *N. tabacum* (Goodspeed, 1954). In contrast, Jamet *et al.* (1987) found that many *N. sylvestris* bands were present in *N. tabacum*. It is possible that the probed region was in that part of the DNA derived from the other parent of *Nicotiana tabacum*, which is thought to have been *N. tomentosiformis* (Sheen, 1971), or that sufficient genome evolution has occurred since the hybridisation event for the *N. sylvestris* sequence to be undetectable at this stringency. An alternative explanation could be that insufficient DNA was present in the *N. sylvestris* track, or that the sample was incompletely digested.

The low number of bands detected in tracks other than of *Nicotiana tabacum* DNA makes it unwise to lay importance on the estimates of relatedness between pairwise combinations of species *not* involving *N. tabacum*. For example, both *N. megalosiphon* and *N. debneyi* appear, from these results, to be more closely related to *N. tabacum* than to each other, although they are both classified as members of the Suaveolentes i.e. the Australian section of the *Nicotiana* genus (Goodspeed, 1954). Interestingly, though, following restriction analysis of the chloroplast genome, Salts *et al.* (1984) also came to the conclusion that *N. debneyi* resembles the South American species more than the other

TABLE 18. Measurement of similarity of band patterns between *Nicotiana* species

From Figure 7, the total number of bands given for each of 6 *Nicotiana* species in an *Eco* RI digest probed with a 2.6kb low copy number probe was counted, and the number of bands shared between each possible pairwise combination of species was noted.

SPECIES	TOTAL NO. OF BANDS	% OF BANDS SHARED BY					
		DEB.	MEG.	RUS.	SYL.	TAB.(WB)	TAB.(X)
N. debneyi	8	-	25.0	25.0	0	50.0	50.0
N. megalosiphon	4	50.0	-	0	0	25.0	75.0
N. rustica	3	66.6	0	-	0	66.6	33.3
N. sylvestris	1	0	0	0	-	0	0
N. tabacum (WB)	7	57.2	14.3	28.6	0	-	71.4
N. tabacum (X)	12	33.3	25.0	8.3	0	41.7	-

Abbreviations: DEB.= *Nicotiana debneyi*, MEG.= *N. megalosiphon*, RUS.= *N. rustica*, SYL.= *N. sylvestris*, TAB.= *N. tabacum*, (WB= White Burley, X= Xanthi)

Australian species tested. From examination of trichome types, Goodspeed (1954) concluded that all members of the Suaveolentes arose from three amphidiploids, now represented by *N. debneyi*, *N. fragrans* and *N. suaveolens*, and that the ancestral diploids came from the families Alatae and Acuminatae (for *N. debneyi*) or Alatae and Noctiflorae (for the other two species). *Nicotiana megalosiphon* is thought to have arisen as a direct derivative of a hybrid between *N. suaveolens* and *N. fragrans* (Goodspeed, 1954). From my results, it appears that *N. debneyi* has retained its South American characteristics despite having a very similar distribution to that of *N. megalosiphon*. More information would be obtained by reducing the stringency of the wash conditions.

These results show that RFLP analysis does have potential applications in studies of the taxonomy and evolution of tobacco.

4.4.3.2 Comparison of the abilities of different restriction enzymes to detect RFLPs between species

This experiment aimed to investigate the abilities of a number of restriction enzymes to differentiate between species of *Nicotiana*.

DNA from *Nicotiana debneyi, N. glutinosa, N. megalosiphon, N. rustica, N. sylvestris and N. tabacum* (cultivars 'Xanthi' and 'White Burley') was digested with 10 restriction enzymes, 5 having 4 base-pair recognition sites (*Alu*I, *Hae*III, *Hpa*II, *Msp*I and *Sau*3AI) and 5 having 6 base-pair recognition sites (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I and *Pst*I). The DNA of *N. debneyi, N. megalosiphon* and *N. rustica* did not cut well with any of these enzymes, and *Pst*I failed to cut any of the DNA samples. These samples were therefore omitted from subsequent gels. Southern blots were prepared from the remaining samples and probed with the same probe used in Section 4.4.3.1. In each case, there was very little or no hybridisation to *N. sylvestris* or *N. glutinosa* DNA, whereas both *N. tabacum* samples hybridised well to the probe. Equal quantities of DNA were loaded in each track, suggesting that there is genuinely less hybridisation to the *N. sylvestris* and *N. glutinosa* samples.

Since band patterns were obtained only for the *N. tabacum* samples, these results are presented and discussed in the section on cultivar identification (Section 4.4.3.3). The experiment was repeated *omitting the final high stringency wash* in an attempt to reveal the DNA in the other tracks, but the patterns obtained were identical to the original ones. This suggests that these hybridisation conditions are highly stringent even without the final wash.

An identical filter was probed with a 1.1kb, highly repeated sequence (#303). The results are summarised in Table 19 and illustrated in Plate 16. Although a high stringency wash was used, this time there was strong hybridisation to *N. glutinosa* and *N. sylvestris* DNA, as well as to *N. tabacum* DNA. The same amounts of DNA were loaded as in the previous experiment, so the explanation for the discrepancy may be that the two probed regions have diverged to different extents. This highlights the importance of combining information from several probes in order to estimate relatedness.

*Bam*HI produced only very large fragments, indicating that it does not cut within the probed region. *Kpn*I degraded the DNA samples, suggesting that it was contaminated with non-specific DNases.

From Table 19, it can be seen that the ability to detect RFLPs in this probed region depended on the restriction enzyme used. For example, with *Msp*I, all except one possible combination of samples could be distinguished whereas all samples appeared identical in a *Sau*3AI digest (Plate 16A). It must be borne in mind that *Msp*I is sensitive to methylation of the 5' cytosine residue in its recognition sequence ($^{5'}$ CCGG $^{3'}$) (Nelson and McClelland, 1989); since this base is in a CXG triplet (where X is any nucleotide), it could be prone to methylation (Gruenbaum *et al.*, 1981), and therefore the differences revealed could be due to differences in in methylation pattern rather than to genetic variation. *Hpa*II, which has the same recognition site as *Msp*I, is sensitive to methylation at *both* cytosine residues, and comparison of *Msp*I and *Hpa*II digests would therefore give some indication of the extent of methylation. Unfortunately, there was poor hybridisation to the *Hpa*II-digested DNA, and so this comparison cannot be made. It is interesting to note, nevertheless, that *N. tabacum* 'Xanthi' DNA could not be cut successfully with *Hpa*II, whereas *Msp*I digestions worked successfully. This suggests that cytosine methylation in CG pairs is extensive in 'Xanthi'. Alternatively, the incomplete digestion may have resulted from higher levels of contamination of the 'Xanthi' DNA with impurities that inhibit digestion: all other restriction enzymes used successfully digested 'Xanthi' DNA, though, so there is no evidence that this latter hypothesis is correct.

The data from Table 19 can also give some information about the relatedness of the 4 samples tested. For example, only one restriction enzyme of the 6 that gave analysable band patterns with samples 1 and 4 could distinguish between these two samples. This is, perhaps, not surprising since these two

TABLE 19. Comparison of different restriction enzymes for screening for RFLPs between *Nicotiana* species

DNA from three *Nicotiana* species (*N. glutinosa, N. sylvestris* and *N. tabacum* cvs 'White Burley' and 'Xanthi') was digested with 8 restriction enzymes and probed with a high copy number, 1.1kb probe (#303). The table shows the results of comparison of the band patterns obtained for each possible pairwise combination of samples.

RESTRICTION ENZYME			distinguishes between						TOTAL NO. OF +	TOTAL NO. OF -	% POSITIVE
			1/2	1/3	1/4	2/3	2/4	3/4			
4 base-pair recognition site	*Alu*	I	?	?	-	?	?	?	0	1	0
	Hae	III	+	-	-	-	+	+	3	3	50.0
	Hpa	II	?	?	n/a	?	n/a	n/a	n/a	n/a	n/a
	Msp	I	+	+	?	+	+	+	5	0	100
	Sau	3AI	-	+	-	+	-	+	3	3	50.0
6 base-pair recognition site	*Bam*	HI	?	?	-	?	?	?	0	1	0
	Eco	RI	?	?	+	?	?	?	1	0	100
	Hin	dIII	+	+	-	-	+	+	4	2	66.6
TOTAL NO. OF +			3	3	1	2	3	4	16		
TOTAL NO. OF -			1	1	5	2	1	0		10	
% POSITIVE			75.0	75.0	16.6	50.0	75.0	100.0			61.5

1= *Nicotiana tabacum* cv 'White Burley', 2= *N. glutinosa* , 3= *N. sylvestris*, 4= *N. tabacum* cv 'Xanthi'

+ distinguishes between these 2 samples
- does not distinguish between these two samples
? not possible to determine (poor or no hybridisation in 1 or more tracks)
n/a not applicable (sample 4 missing)

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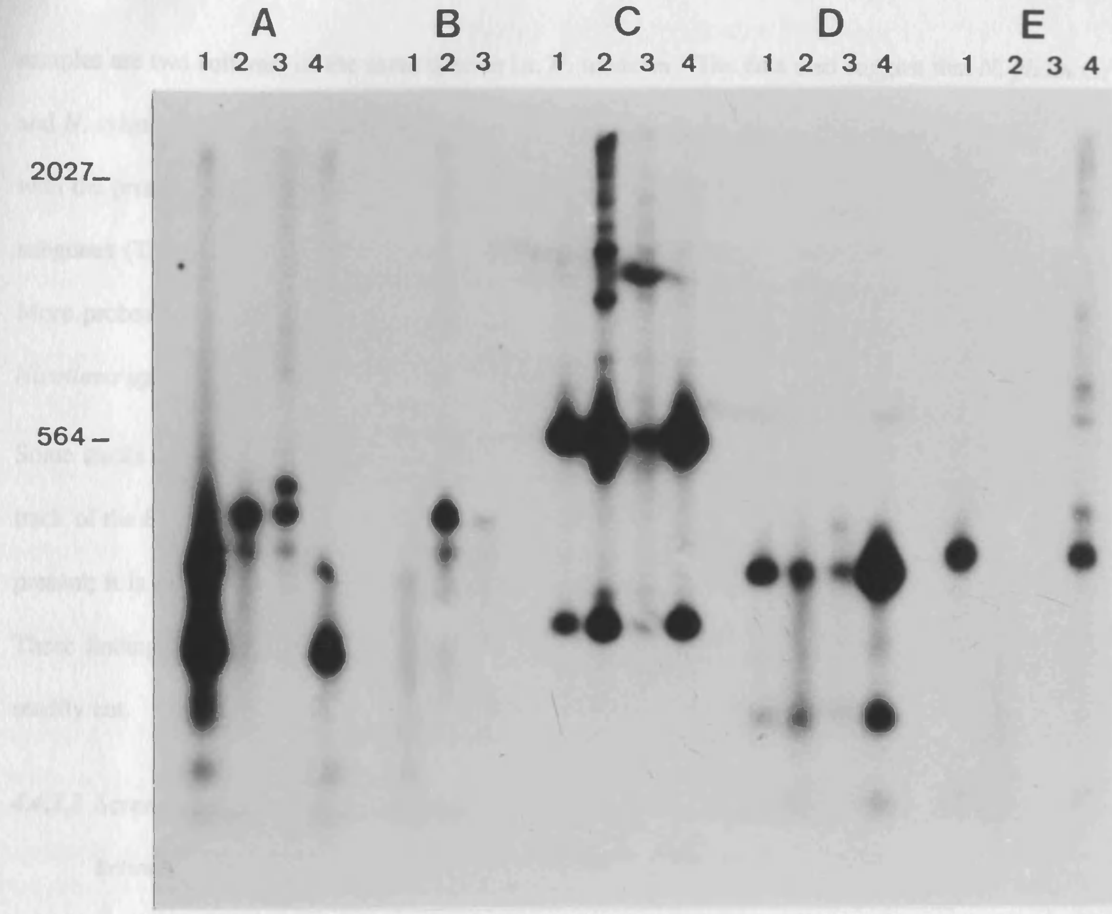
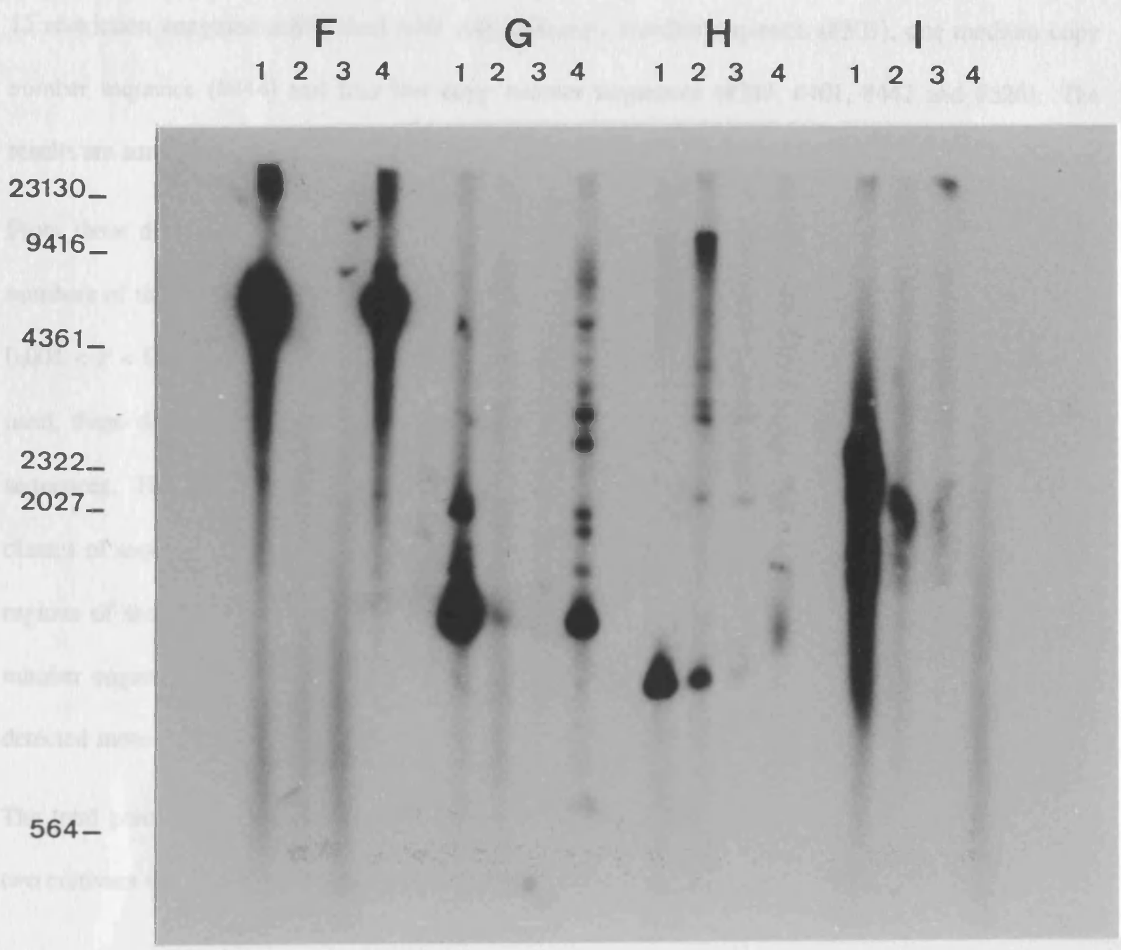
Plate 16:

Restriction digests of DNA from different species and cultivars of *Nicotiana* (1= *Nicotiana tabacum* c.v. 'White Burley', 2= *N. glutinosa* , 3= *N. sylvestris* , 4= *N. tabacum* c.v. 'Xanthi') probed with a 1.1kb, high copy number sequence (#303).
(A) *Msp* I, (B) *Hpa* II, (C) *Hae* III, (D) *Sau* 3A1, (E) *Alu* I I
(F) *Bam* HI, (G) *Eco* RI, (H) *Hin* dIII, (I) *Kpn* I


A
B
C
D
E
1 2 3 4
1 2 3
1 2 3 4
1 2 3 4
1 2 3 4
2027
564


F
G
H
I
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4
23130
9416
4361
2322
2027
564

samples are two cultivars of the same species i.e. *N. tabacum*. The data also suggest that *N. glutinosa* and *N. sylvestris* are more closely related to each other than to *N. tabacum*. This is not in agreement with the present classification of *Nicotiana* species: both *N. tabacum* and *N. glutinosa* are in the same subgenus (Tabacum), whereas *N. sylvestris* belongs to the subgenus Petunioides (Goodspeed, 1954). More probes would have to be used in order to obtain more information on the phylogeny of these *Nicotiana* species.

Some tracks on these gels contained several high molecular weight bands, for example, the 'Xanthi' track of the *Eco*RI digest (Plate 16B). In a replicate of this digestion (Plate 17), these bands were not present; it is therefore suggested that these bands are the products of incomplete restriction digestion. These findings emphasis the importance of replication in experiments involving DNA that does not readily cut.

4.4.3.3 Screening different restriction enzyme/ probe combinations for their ability to detect RFLPs between cultivars

DNA from two cultivars of *Nicotiana tabacum* — 'Xanthi' and 'White Burley'— was digested with 15 restriction enzymes and probed with one high copy number sequence (#303), one medium copy number sequence (#444) and four low copy number sequences (#317, #401, #442 and #526). The results are summarised in Table 20 and examples of band patterns are shown in Plates 17-19.

From these data, it was calculated that there was a statistically significant difference between the numbers of times that RFLPs were detected between the three different sequence classes (χ^2= 9.642, $0.001 < P < 0.01$), the single-copy sequences being superior. As only small numbers of probes were used, these data may not be representative of the true level of variation in the different types of sequences. However, if these results are an accurate reflection of the variability of the different classes of sequence, then they are surprising: single copy sequences are thought to be mostly coding regions of the DNA and would therefore be expected to be more conserved than the higher copy number sequences. In agreement with these results, Landry *et al.*, (1987) found that cDNA clones detected more RFLPs in lettuce than did genomic clones.

The total percentage of restriction enzyme/probe combinations that revealed RFLPs between these two cultivars was 35.2%; this figure cannot be cited as the "relatedness" of the two cultivars, though,

TABLE 20. Screening for RFLPs between cultivars

Different restriction enzyme/probe combinations were tested for their abilities to detect RFLPs between two cultivars of *Nicotiana tabacum*, 'Xanthi' and 'White Burley'.

RESTRICTION ENZYME	RECOGNITION SITE	PROBE						TOTAL NO. OF +	TOTAL NO. OF -	% POSITIVE
		HIGH COPY NO.	MEDIUM COPY NO.	LOW COPY NO.						
		#303	#444	#317	#401	#442	#526			
Alu I	AGCT	-	-	+	NH	-	-	1	4	20
Bst UI	CGCG	-	not cut	n/a	NH	-	NH	1	4	20
Hae III	GGCC	n/a	n/a	+(C)	n/a	n/a	n/a	1	0	100
Hpa II	CCGG	-	not cut	NH	NH	-	NH	0	2	0
Mnl I	CCTC	-	-	n/a	NH	-	NH	0	3	0
Msp I	CCGG	-	-	n/a	NH	-	NH	0	3	0
Rsa I	GTAC	-	+	n/a	NH	+	+	3	1	75
Sau 3AI	GATC	-	-	+	NH	-	-	1	4	20
Bam HI	GGATCC	-	-	+	NH	-	-	2	3	40
Bgl II	AGATCT	-	-	n/a	NH	-	+	1	3	25
Bsc I	ATCGAT	+	not cut	n/a	NH	+	+	3	0	100
Dra I	TTTAAA	-	-	-	NH	+	-	1	3	25
Eco RI	GAATTC	+	-	+	NH	+	+	4	1	80
Hin dIII	AAGCTT	-	-	-	NH	+	+	2	3	40
Sma I	CCCGGG	-	not cut	n/a	NH	-	-	0	3	0
TOTAL NO. OF +		2	1	5	0	5	6	19		
TOTAL NO. OF -		12	9	1	0	9	4		35	
% SUCCESS		14.3	10.0	83.3	n/a	35.7	60.0			54.3

+ RFLP(s) detected
- no variation
* possible partial digestion
C copy number change
NH no hybridisation
n/a not applicable

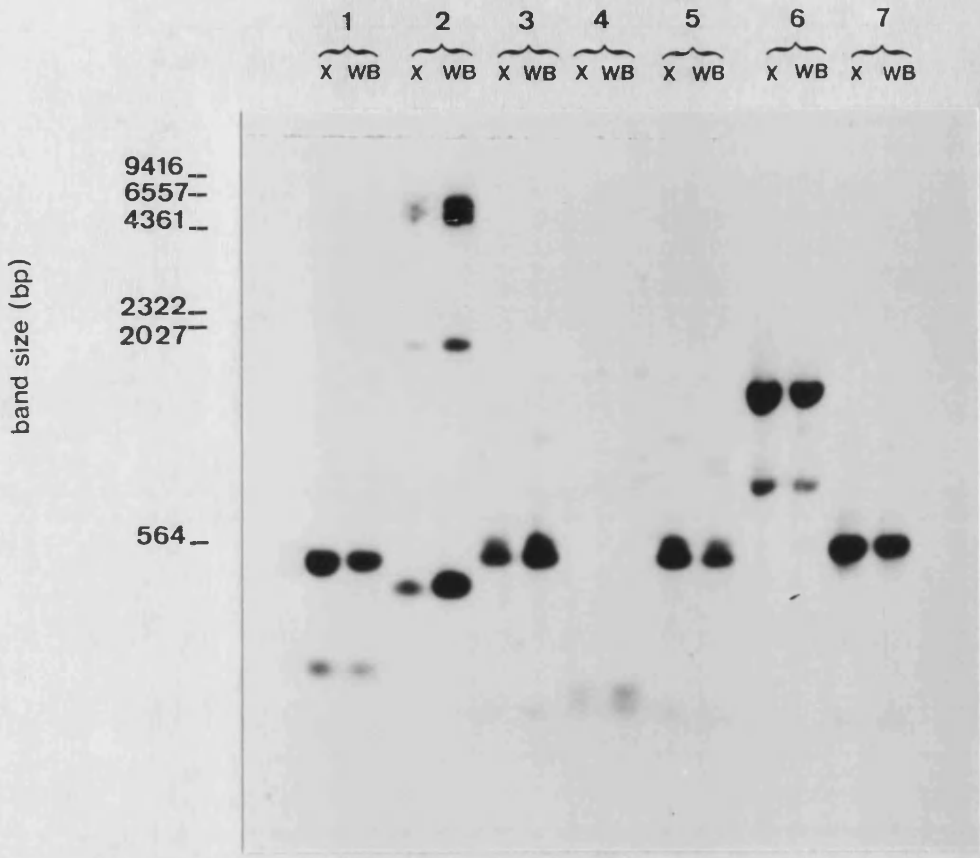
facing page 113

Plate 17: Screening for RFLPs between cultivars of *Nicotiana tabacum* . (I)

Restriction digests of *Nicotiana tabacum* DNA (X= Xanthi, WB= White Burley) probed with a 1.1kb, high copy number sequence (#303).

Above: (1) *Alu* I, (2) *Bst* UI, (3) *Hpa* II, (4) *Mnl* I, (5) *Msp* I, (6) *Rsa* I, (7) *Sau* 3AI.

Below: (1) *Bam* HI, (2) *Bgl* II, (3) *Bsc* I, (4) *Dra* 1, (5) *Eco* RI, (6) *Hin* dIII, (7) *Sma* I.


1
2
3
4
5
6
7
X WB X WB X WB X WB X WB X WB X WB
band size (bp)
9416
6557
4361
2322
2027
564

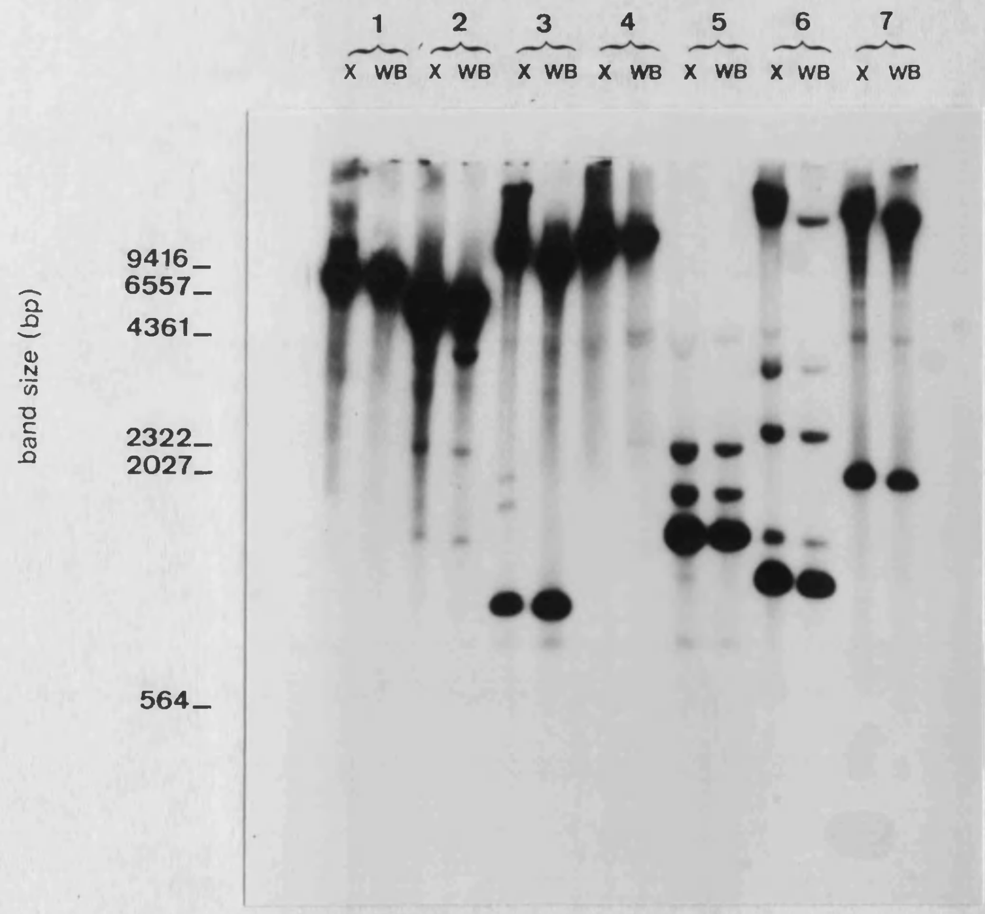

1
2
3
4
5
6
7
X WB X WB X WB X WB X WB X WB X WB
band size (bp)
9416
6557
4361
2322
2027
564

Plate 18: Screening for RFLPs between cultivars of *Nicotiana tabacum* . (II)

Restriction digests of *Nicotiana tabacum* DNA (X= Xanthi, WB= White Burley) probed with a 1.55kb, medium copy number sequence (#444).

Above: (1) *Alu* I, (2) *Bst* UI, (3) *Hpa* II, (4) *Mnl* I, (5) *Msp* I, (6) *Rsa* I, (7) *Sau* 3AI.

Below: (1) *Bam* HI, (2) *Bgl* II, (3) *Bsc* I, (4) *Dra* 1, (5) *Eco* RI, (6) *Hin* dIII, (7) *Sma* I.

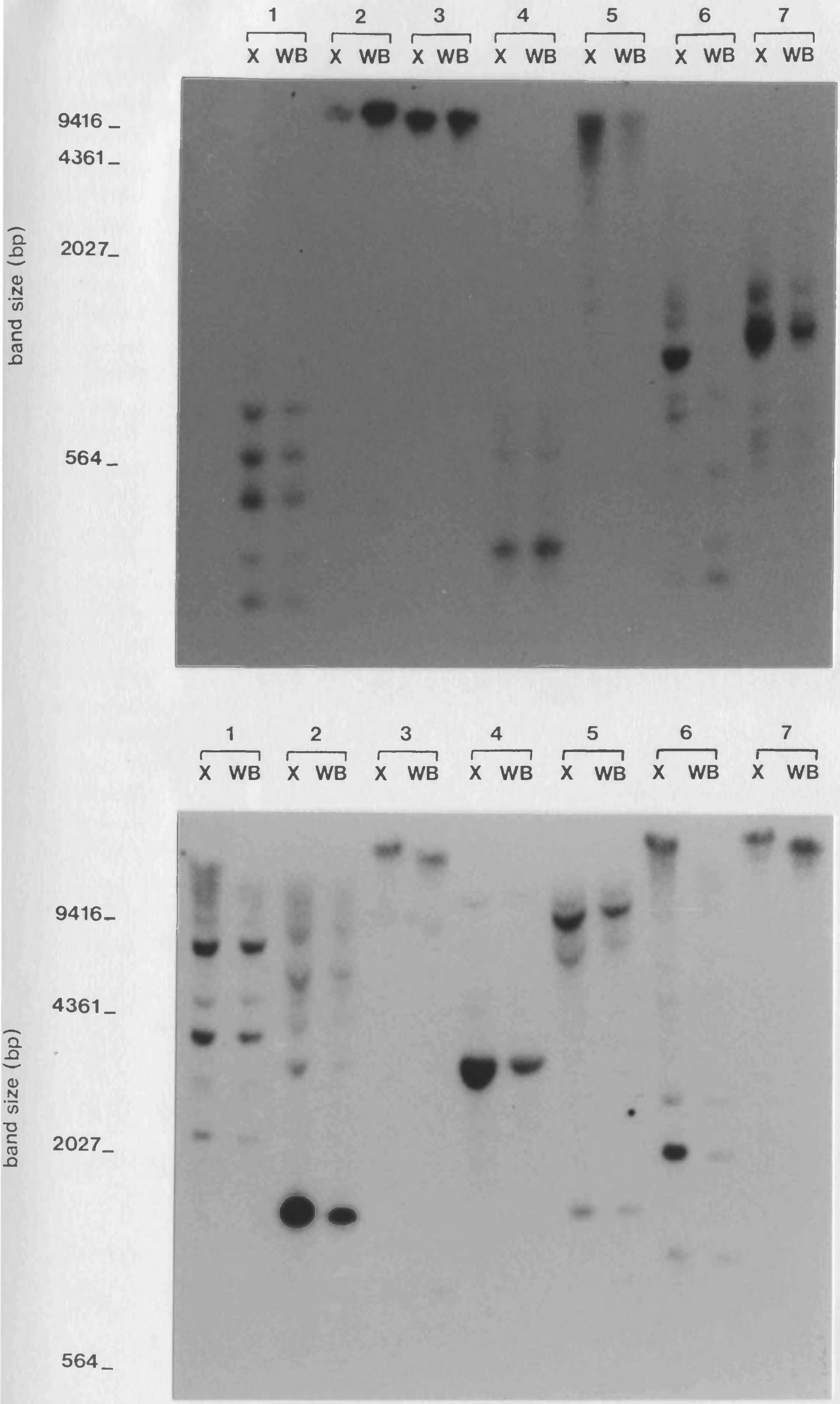
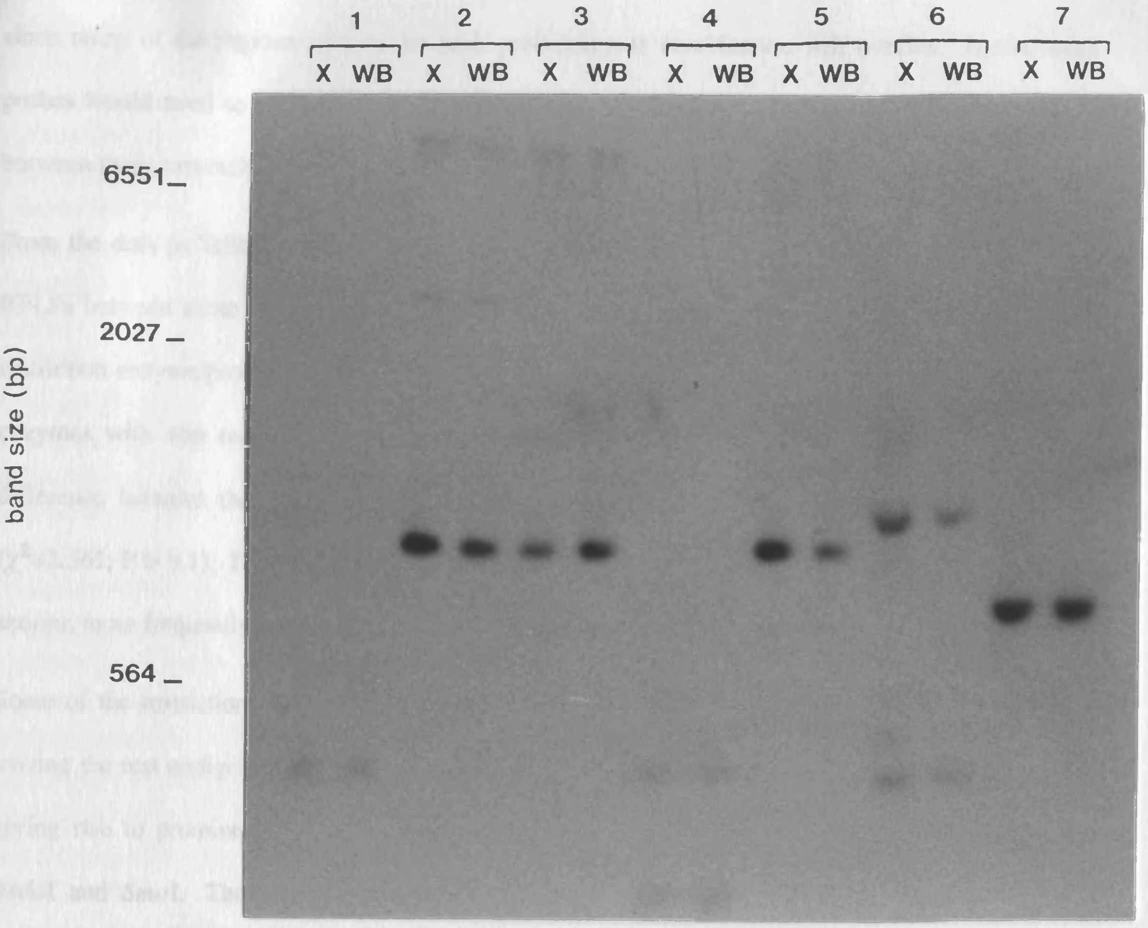
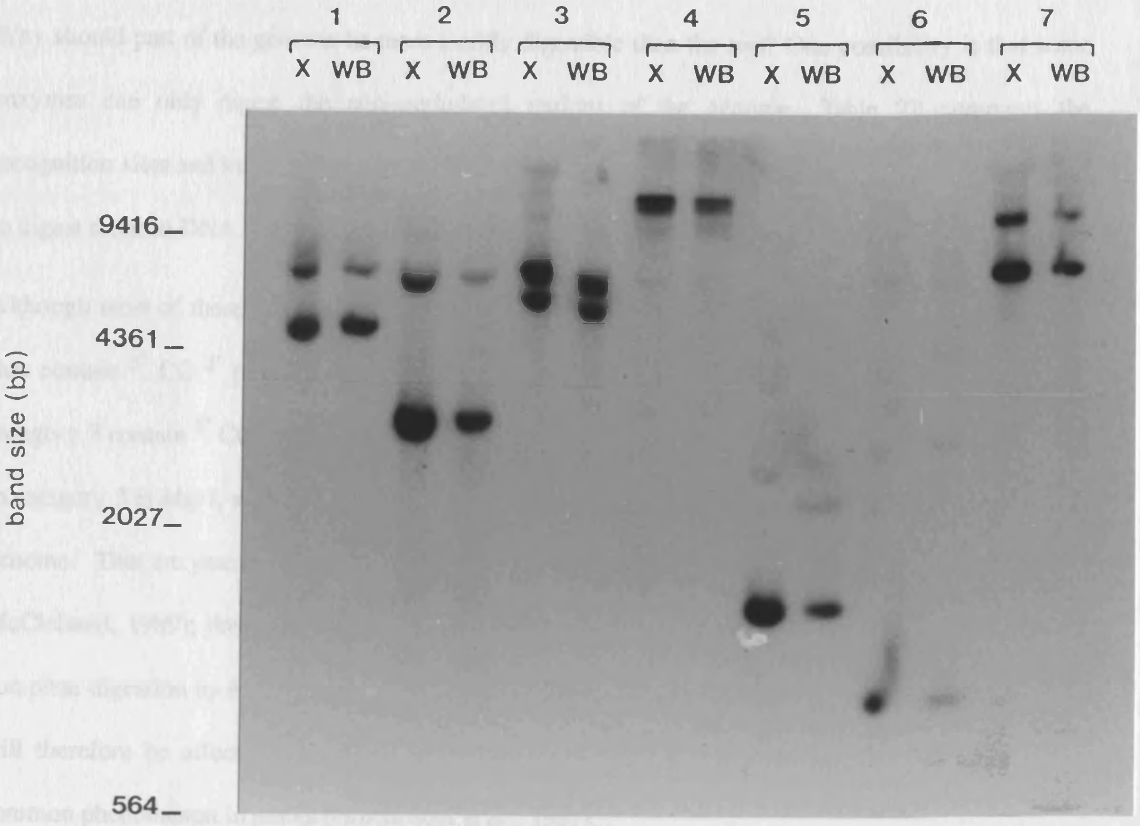

1 2 3 4 5 6 7
X WB X WB X WB X WB X WB X WB X WB
9416
4361
2027
564
band size (bp)
1 2 3 4 5 6 7
X WB X WB X WB X WB X WB X WB X WB
9416
4361
2027
564
band size (bp)

Plate 19: Screening for RFLPs between cultivars of *Nicotiana tabacum* . (III)

Restriction digests of *Nicotiana tabacum* DNA (X= Xanthi, WB= White Burley) probed with a 1.05kb, low copy number sequence (#442).
Above: (1) *Alu* I, (2) *Bst* UI, (3) *Hpa* II, (4) *Mnl* I, (5) *Msp* I, (6) *Rsa* I, (7) *Sau* 3AI.

Below: (1) *Bam* HI, (2) *Bgl* II, (3) *Bsc* I, (4) *Dra* 1, (5) *Eco* RI, (6) *Hin* dIII, (7) *Sma* I.


1
2
3
4
5
6
7
X WB X WB X WB X WB X WB X WB X WB
6551
2027
564
band size (bp)


1
2
3
4
5
6
7
X WB X WB X WB X WB X WB X WB X WB
9416
4361
2027
564
band size (bp)

since many of the regions covered by each probe/enzyme ombination will overlap. Again, more probes would need to be used in order to make a more acarate estimate of the overall variation between these two cultivars.

From the data in Table 20, the restriction enzymes were ranled according to their ability to detect RFLPs between these two cultivars. The ranking is shown it Table 21. The overall percentages of restriction enzyme/probe combinations that detected RFLPs beween these two cultivars were 24% for enzymes with 4bp recognition sequences, and 44.8% for those with 6bp recognition sites. This difference between the two different classes of enzymes wa not, however, statistically significant (χ^2=2.561, $P > 0.1$). Despite the theoretical extra coverage ofthe genome afforded by enzymes with shorter, more frequently occurring recognition sites, then, such enzymes had no apparent advantage.

Some of the restriction enzymes used in this experiment appared to cut only part of the genome, leaving the rest undigested. The portion that was digested, though, seemed to be completely digested, giving rise to prominent bands (Plate 20) This phenomenon was especially noticeable with *Bsc*I, *Bst*UI and *Sma*I. The DNA appeared to have digested to completion with other enzymes, for example, *Alu*I and *Dra*I.

Why should part of the genome be more readily digestible thai the rest? One possibility is that some enzymes can only digest the non-methylated regions of tie genome. Table 22 compares the recognition sites and known methylation sensitivities of the resriction enzymes used with their ability to digest tobacco DNA.

Although most of these enzymes are sensitive to methylation of particular bases, it is those enzymes that contain $^{5'}$ CG $^{3'}$ pairs that fail to digest the tobacco DNA completely—all of the enzymes in category 3 contain $^{5'}$ CG $^{3'}$ pairs. The only enzyme that has a ste containing a $^{5'}$ CG $^{3'}$ pair and is *not* in category 3 is *Msp*I, which has the same recognition site as *Hpa*II but was able to digest more of the genome. This enzyme is not sensitive to methylation of the nternal cytosine residue (Nelson and McClelland, 1989); this may explain why it can digest more of the genome than *Hpa*II. The lack of complete digestion by *Msp*I may be because it is sensitive to methylation of the 5' cytosine residue. It will therefore be affected by methylation in some $^{5'}$ CXG $^{3'}$sequences, which is thought to be a common phenomenon in plants (Gruenbaum *et al.*, 1981).

TABLE 21. Ranking of restriction enzymes according to their ability to detect RFLPs between cultivars

The data from Table 20 were used to rank the enzymes used according to their ability to detect RFLPs between *Nicotiana tabacum* cultivars 'White Burley' and 'Xanthi'.

% OF TIMES VARIATION DETECTED	RESTRICTION ENZYME	RECOGNITION SITE 5' 3'
0	*Bst* UI	CGCG
	Hpa II	CCGG
	Mnl I	CCTC
	Sma I	CCCGGG
20	*Alu* I	AGCT
	Sau 3AI	GATC
25	*Bgl* II	AGATCT
	Dra I	TTTAAA
40	*Bam* HI	GGATCC
	Hin dIII	AAGCTT
75	*Rsa* I	GTAC
80	*Eco* RI	GAATTC
100†	*Hae* III	GGCC
	Bsc I	ATCGAT

† based on a single probe/enzyme combination

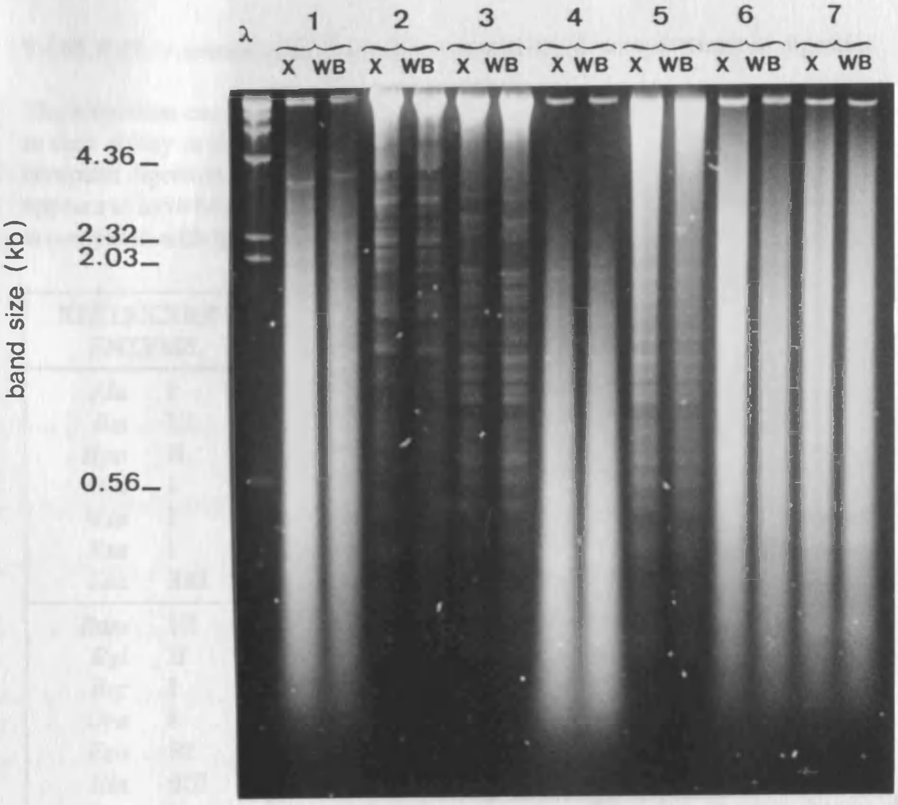
facing page 118

Plate 20:

DNA from Nicotiana tabacum cultivars 'Xanthi' (X) and 'White Burley' (WB) following restriction digestion and gel electrophoresis.

Above: (1) *Alu* I, (2) *Bst* UI, (3) *Hpa* II, (4) *Mnl* I, (5) *Msp* I, (6) *Rsa* I, (7) *Sau* 3AI.

Below: (1) *Bam* HI, (2) *Bgl* II, (3) *Bsc* I, (4) *Dra* 1, (5) *Eco* RI, (6) *Hin* dIII, (7) *Sma* I.


λ
1 2 3 4 5 6 7
X WB X WB X WB X WB X WB X WB X WB
4.36
2.32
2.03
0.56
band size (kb)

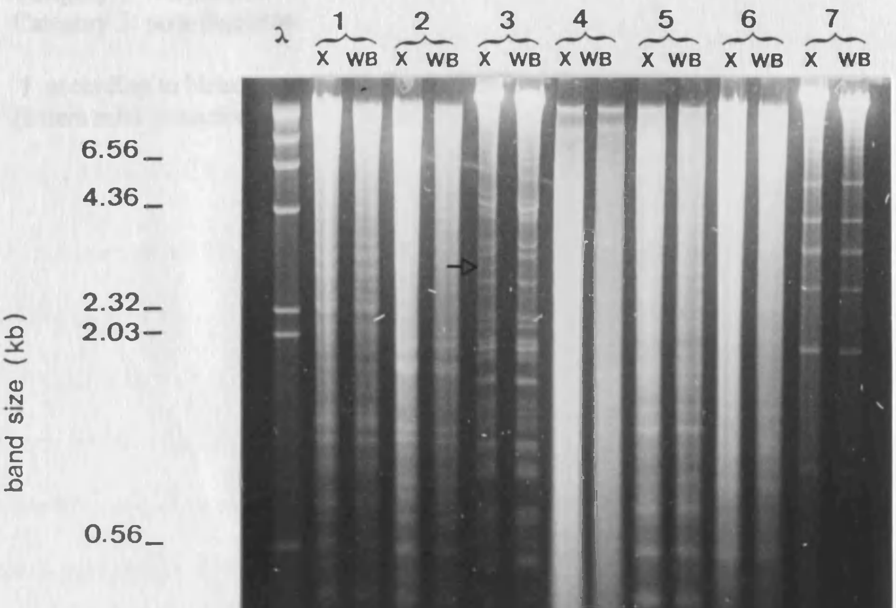

λ
1 2 3 4 5 6 7
X WB X WB X WB X WB X WB X WB X WB
6.56
4.36
2.32
2.03
0.56
band size (kb)

TABLE 22. Association of methylation sensitivity & completeness of digestion

The restriction enzymes were graded, from the gels shown in Plate 20, according to their ability to digest to completion tobacco DNA. Grade 1 represents complete digestion, through to Grade 3, where only a small fraction of the DNA appears to have been digested. The ability of each restriction enzyme to cut is compared with its recognition site and its known methylation sensitivity.

RESTRICTION ENZYME	RECOGNITION SITE	METHYLATION SENSITIVITY†	EXTENT OF DIGESTION
Alu I	AGCT	A1 C3	1
Bst UI	CGCG	C1 C3	3
Hpa II	CCGG	C1 C2	3
Mnl I	CCTC	C1	1
Msp I	CCGG	C1	2
Rsa I	GTAC	none	1
Sau 3AI	GATC	C4	1
Bam HI	GGATCC	C5	2
Bgl II	AGATCT	C5	2
Bsc I	ATCGAT	not known	3
Dra I	TTTAAA	none	1
Eco RI	GAATTC	A2 A3 C6	2
Hin dIII	AAGCTT	A1 C4	1
Sma I	CCCGGG	C3	3

Category 1: complete digestion
Category 2: intermediate
Category 3: poor digestion

† according to Nelson and McClelland (1989)
(letters refer to nucleotide, and numbers to position in sequence)

These observations are in agreement with the hypothesis that methylation, of cytosine residues in $^{5'}$ CG $^{3'}$ and $^{5'}$ CXG $^{3'}$ sequences, was the cause of the incomplete digestion observed. The part of the genome that does cut must, therefore, be all the non-methylated regions, which include active genes (Van der Ploeg and Flavell, 1980), chloroplast DNA (Ngernprasirtsiri *et al.*, 1988) and mitochondrial DNA (Ward *et al.*, (1981). Since active genes are usually present only in low copy number (Vedel and Delseny, 1987), the highly repeated, prominent bands seen in the partial digests are probably, therefore, chloroplast DNA.

The complete chloroplast DNA sequence of the *Nicotiana tabacum* cultivar 'Bright Yellow' has been determined (Shinozaki *et al.*, 1986) and deposited with the GenBank database. The University of Wisconsin Computer Group programmes (version 6) (Devereux *et al.*, 1984) were used to determine the sizes of fragments that would be produced by *Sma*I digestion of this chloroplast DNA sequence; a comparison of these predicted sizes with the actual fragment sizes obtained with *Sma*I is given in Table 23. It is clear from these data that the prominent bands are indeed chloroplast DNA sequences: for each band observed, there is a predicted band very close in size. In two cases (the 8913 bp and 3500 bp bands), the observed band must have been 2 closely migrating fragments that could not be resolved using this electrophoresis system. The predicted 51 bp band would not have been detectable, and the very high molecular weight fragments would have been indistinguishable from the high molecular weight, undigested nuclear DNA at the top of the track (Plate 20).

These results indicate that much of the tobacco *nuclear* genome is methylated. Since methylation is associated with a lack of transcriptional activity (see review by Doerfler, 1983), it seems that a large proportion of the tobacco genome is "switched off". This is in agreement with the results of Goldberg *et al.* (1978)—they estimated that about 15,000 different mRNAs were expressed in *Nicotiana tabacum* ; this corresponds to about 1.8×10^4 kb of the genome. It has been suggested that all higher plants have approximately the same number of coding genes and that the vast differences in genome size between different plant species (ranging from 0.4pg per diploid cell in *Arabidopsis thaliana* to 98.1pg in *Hyacinthus orientalis* (Vedel and Delseny, 1987)) (the "C-value paradox" (Cavalier-Smith, 1985)) are due largely to differences in the quantity of "selfish" DNA rather than to variation in the number of active genes. Such "selfish" DNA is thought to accumulate because of its ability to replicate rather than because it has a specific function (Doolittle and Sapienza, 1980).

TABLE 23. Comparison of sizes of observed and predicted *Sma* I fragments of chloroplast DNA

The sizes of highly repeated DNA fragments observed after *Sma* digestion of total genomic DNA are compared with the predicted sizes of fragments produced by *Sma* I digestion of chloroplast DNA.

OBSERVED SIZE (bp)	PREDICTED SIZE (bp)
not possible to determine	26521 22638 16342 11126 9925
8913	8913
	8778
7585	7537
5754	5459
4216	4014
3500	3343
	3295
2600	2638
1840	1843
916	929
	51

Since a large proportion of the tobacco genome is methylated, restriction enzymes that are sensitive to methylation, particularly those with CG pairs in their recognition sites, are unsuitable for the analysis of genetic variation in the nuclear DNA of tobacco. Henceforth, therefore, such enzymes were avoided. The inability of these enzymes to digest the majority of the nuclear DNA could, though, be exploited by using them to study the chloroplast genome. This could be useful for screening large numbers of samples without having to isolate highly pure samples of chloroplast DNA—any contamination with nuclear DNA would not be a problem.

Examination of the chloroplast DNA bands produced by *Bsc*I (Plate 20) shows that a 2.9kb band in 'Xanthi' is absent from 'White Burley' (arrowed). This may represent an insertion or deletion, but precise determination of the nature of the change will require repetition of the gel to improve the resolution.

It is possible to make some inferences, from the hybridisation patterns observed using different probes on these incompletely digested gels, concerning the nature of the probed sequences. All single copy sequences hybridised to discrete bands, and not to high molecular weight fragments. This indicates that none of these sequences was methylated, suggesting that each was actively transcribed in the leaf. In contrast, the medium copy number sequence used hybridised only to high molecular weight bands in restriction digests with *Bst* UI, *Hpa* II, *Msp* II, and *Sma*I. With the exception of *Hin* dIII, these enzymes all have restriction sites containing a CG sequence, and are all sensitive to methylation. With other enzymes, hybridisation was to lower molecular weight fragments. This evidence suggests that this sequence is methylated. The high copy number sequence hybridised to both high and lower molecular weight bands in some digests, suggesting that some copies of the sequence are methylated and others not. One possible explanation for this is that some copies are in the chloroplast and others in the nucleus; colony hybridisation suggested that this might be the case (Section 4.4.2.1) but no region with a similar restriction map could be found in the known sequence of the tobacco chloroplast genome (Shinozaki *et al.*, 1986). An alternative hypothesis is that all copies are nuclear in origin, but that some are non-methylated; for example, this could be a multi-gene family with some sequences temporarily or permanently "switched off".

Three restriction enzymes that readily digest tobacco DNA to completion, *Alu*I, *Eco*RV and *Taq*I, were used to digest the DNA from 6 cultivars of *Nicotiana tabacum*. The digested DNA was probed with two low copy number sequences, #443 and #526.

The only probe/enzyme combination that revealed RFLPs between these cultivars was that of probe number 526 and a *Taq*I digestion (Plate 21). Using this combination, 'Xanthi' and 'Samsun' gave qualitatively the same pattern of bands, but the top, 2.48kb band was more intense in 'Samsun'. No hybridisation was detectable in the SR1 track, but from the gel in Plate 21A it can be seen that there was considerably less DNA in this track. The weakness of hybridisation to the 'Virginia' track cannot be explained in the same way, though, since this track contained as much DNA as other tracks. This tobacco variety may have diverged too much from 'Xanthi' to show strong cross-hybridisation under these highly stringent conditions.

It is interesting to note that in both SR1 and the 'Virginia' DNA samples, a single, high molecular weight band (c. 20kb) was seen on the gel itself (Plate 21A, tracks 3 and 6); this band was absent in the other varieties. This suggests that these two varieties may be closely related.

The 'Western' variety gave the same band pattern as 'White Burley'; both lacked the 1.73kb band present in 'Xanthi' and 'Samsun', and had the 2.48kb band as the major fragment (compared to the 0.83kb band in 'Xanthi' and 'Samsun').

From these results, then, the cultivars tested can be divided into 3 pairs of related varieties:

i. SR1 and Virginia

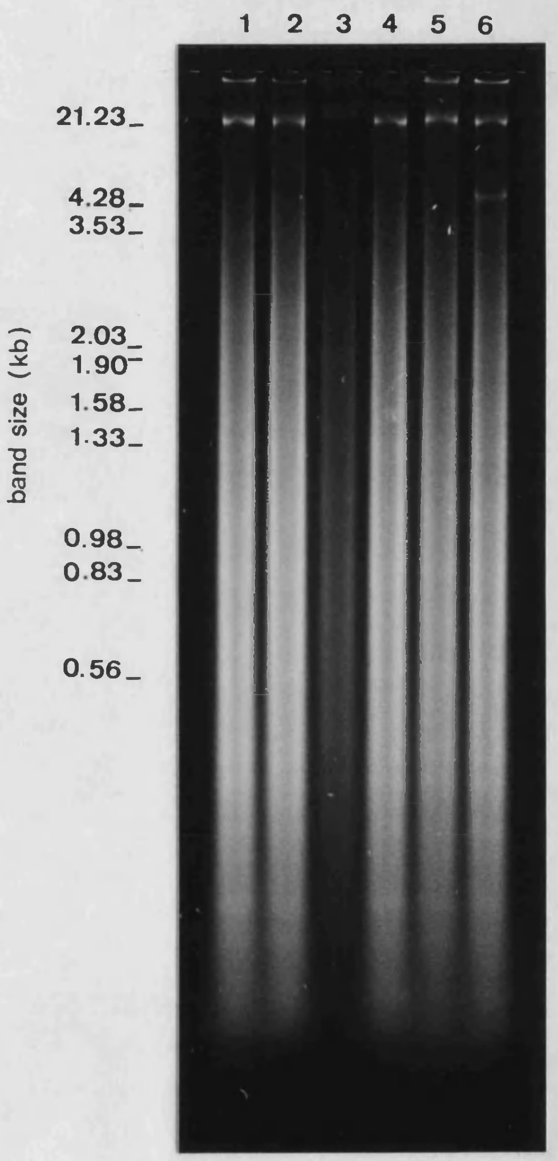
ii. Xanthi and Samsun

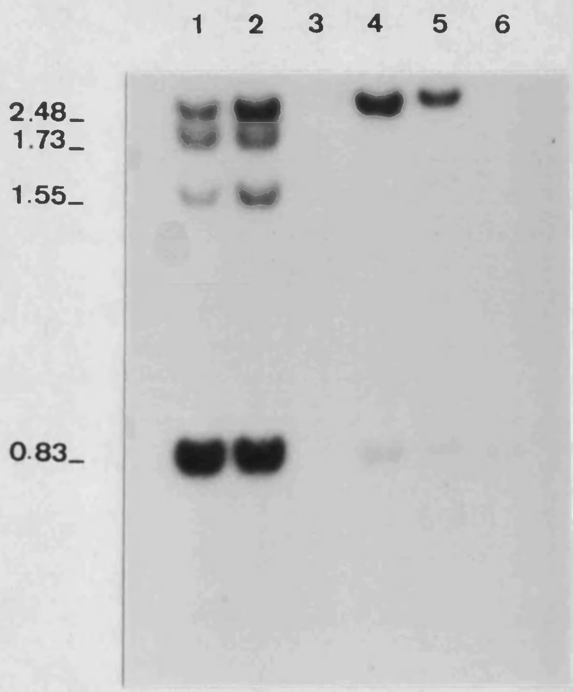
iii. White Burley and Western

Xanthi and Samsun are both so-called 'oriental' tobacco varieties, their cultivation being based around the cities of the same names (in Greece and Turkey respectively); the origin of the oriental tobaccoes is not known, but they may be derived from the initial introduction, probably from America, over 300 years ago (Akehurst, 1968). My results suggest that there is a close relationship between these two varieties, indicating that they came from the same original source.

Plate 21: RFLPs between cultivars of *Nicotiana tabacum*

Left: A *Taq* I digestion of DNA from six cultivars of *Nicotiana tabacum* (1= Xanthi, 2= Samsun, 3= SR1, 4= White Burley, 5= Western, 6=Virginia)
Right: The gel on the left probed with a low copy number, 1.71kb sequence (#526)


1 2 3 4 5 6
21.23
4.28
3.53
2.03
1.90
1.58
1.33
0.98
0.83
0.56
band size (kb)


1 2 3 4 5 6
2.48
1.73
1.55
0.83

Western is a fire-cured tobacco variety known to be introduced to Malawi from Kentucky and Tennessee, but its precise origin is unknown (Akehurst, 1968). The similarity of its restriction sites to those of White Burley (a spontaneous mutant of Burley, which was grown extensively in Kentucky) (Plate 21) may shed some light on the origin of the Western variety.

The results of this experiment illustrate the power of RFLP analysis in taxonomy; they also show the comparitive stability of the tobacco genome.

4.4.3.4 Screening for variation between individual seed-grown plants

In order to investigate the extent of variation between individuals in a seed-grown population of *Nicotiana tabacum*, DNA isolated from 14 'Xanthi' plants was digested with 4 different restriction enzymes (*Alu* I, *Eco* RI, *Hin*dIII and *Taq*I) and probed with one highly repeated sequence (#303), one medium copy number sequence (#444) and two low copy number sequences (#443 and #526). None of the combinations tested revealed RFLPs. An example of an autoradiograph is shown in Plate 22. These preliminary data suggest that there is a high degree of genetic uniformity within a cultivar. This might be expected in a plant that reproduces predominantly by self-fertilisation. Only a tiny proportion of the genome has been examined, however, and so further analyses would be necessary to establish whether there is the same degree of uniformity throughout the genome. These could include screening with many more probes, or with a multi-locus "fingerprinting" probe.

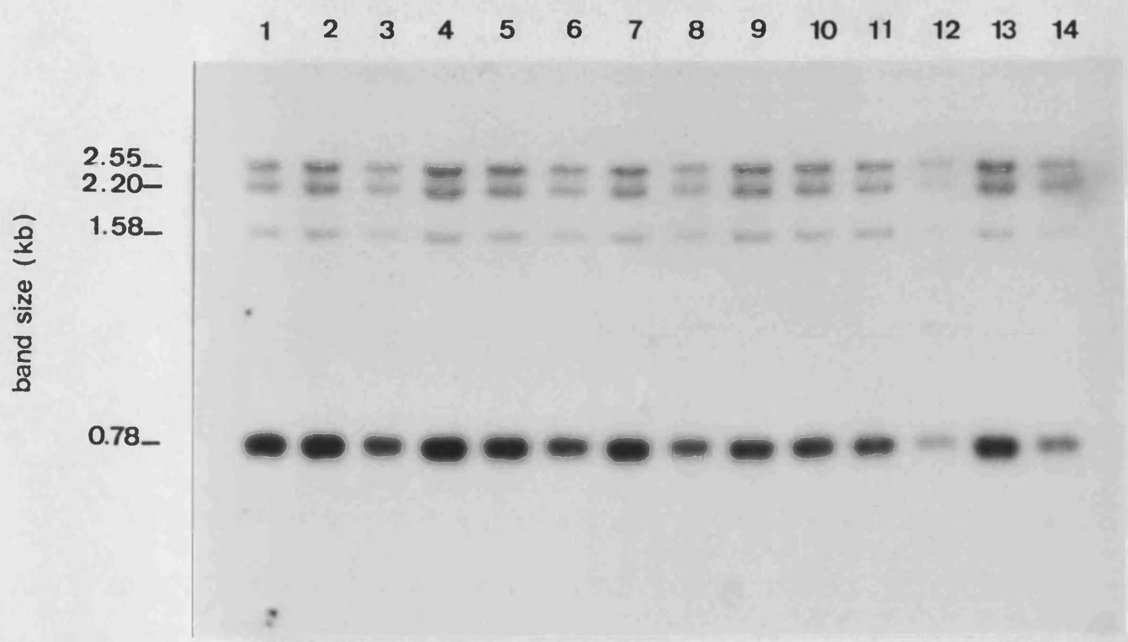
4.4.3.5 Screening for genetic variation within a single plant

An important step in advancing our understanding of the origin of somaclonal variation would be the determination of the extent of genetic variation within the initial explant. As a first step towards this goal, DNA was extracted from different tissues of the same plant (the shoot tip, young leaves, mature leaves, young stem tissue, woody stem tissue and roots), digested with *Alu*I, *Eco*RV and *Taq*I and probed with two low copy number sequences (#443 and #526). There was no evidence of any genetic variation between the different tissues. This is consistent with the current theory that developmental differences are based on different patterns of gene expression rather then on differences in genome organisation (Davidson, 1976).

The power of resolution of these techniques, though, is not sufficient to answer a question of fundamental importance to our understanding of the origin of somaclonal variation i.e. how much

Plate 22:

A *Taq* I digestion of DNA from 14 seed-grown plants, probed with a 1.71kb, low copy number sequence (#526).


1
2
3
4
5
6
7
8
9
10
11
12
13
14
band size (kb)
2.55
2.20
1.58
0.78

genetic variation is there between individual cells in a plant? Somatic mutation has been detected in human cancers (Thein *et al.*, 1987), but a relatively large quantity of homogeneous tissue is required to detect such changes— RFLPs would only be detectable if a large sector of tissue was derived from a somatic mutant cell; any genetic differences present in just one or a few cells would be undetectable, since there would not be enough of the altered DNA sequences on the gel to hybridise to the probe. In the future, it may be possible to investigate the frequency of very small-scale changes by using RFLP analysis in conjunction with the polymerase chain reaction (PCR), which allows the *in vitro* amplification of specific DNA sequences (Saiki *et al.*, 1985). This technique has already allowed forensic scientists to identify criminals from very small samples of cells (Gill *et al.*, 1985) and Li *et al.* (1988) have been able to detect, by dot blotting, DNA from single haploid cells. There does not seem to be any reason why it should not now be possible to produce DNA fingerprints from individual cells (Jeffreys *et al.*, 1988). Isolation of DNA from up to 10 single plant protoplasts has also been achieved (Crossway and Houck, 1985); a combination of this technique with PCR could therefore make it possible to investigate cell-cell variation in plants.

4.4.3.6 Screening somaclones using RFLP analyses

Two batches of 15 regenerated plants from one-step and two-step regeneration systems (Section 2.1.2) were screened for variation using nine restriction enzymes and eight probes. DNA from a control, seed-grown 'Xanthi' plant was included on each gel as a control. The results are summarised in Tables 24 and 25: Table 24 shows the number and the size of bands revealed by each probe/enzyme combination, and Table 25 shows the frequency and nature of variation detected. The lengths and copy numbers of the probe sequences, determined as described in Section 4.3.5.4.5, are indicated. The results obtained are discussed below, with each class of sequence copy number treated separately.

4.4.3.6.1 Highly repeated sequences

From Table 25, it can be seen that probe #303 revealed RFLPs and/or copy number variation in several digests. The nature of the changes seen is not, however, consistent with the hypothesis that this is genetic variation: the same change was sometimes found in several tracks on one gel, in the DNA of plants from unrelated cultures. Examples of this are shown in Plate 23. Either this must represent some very common change, that occurred several times in independent cultures, or it must

TABLE 24. Screening for somaclonal variation using RFLP analyses (1)

This table shows the number of bands detected by each restricion enzyme/probe combination, followed (in brackets) by the total length (in kilobases) of the bands.

RESTRICTION ENZYME	PROBE								TOTAL	MEAN BAND LENGTH (kb)	TOTAL NO. OF PROBES	MEAN NO. OF BANDS PER PROBE
	HIGH COPY NUMBER		MEDIUM COPY NO.	LOW COPY NUMBER								
	#58 (0.95kb) C[a]	#303 (1.10kb) C + N	#444 (1.55kb) N	#308 (1.25kb) N	#442 (1.05kb) N	#443 (1.23kb) C	#526 (1.71kb) N	#604 (0.72kb) N				
Alu I	3† (1.15)	4† (1.80)	7† (5.97)	NH	3 (1.83)	2 (1.80)	4 (2.24)	NH	23 (14.79)	0.64	6	3.83
Hae III	1 (1.04)	3† (0.78)	5 (1.69)	1 (1.08)	3 (1.68)	-	-	NH	13 (6.27)	0.48	5	2.60
Rsa I	4 (1.65)	3 (2.40)	7 (4.41)	NH	3 (2.79) -	-	-		17 (11.25)	0.66	4	4.25
Taq I	2 (1.77)	4† (1.76)	3* (3.32)	1 (0.57)	3 (1.36)	1 (1.35)	4 (6.59)	NH	18 (16.72)	0.93	7	2.57
Bam HI	1† (7.50)	1† (2.82)	6 (24.25)	2 (8.36)	2 (11.34)	-	-	NH	12 (54.27)	4.53	5	2.40
Bgl II	NH	3 (14.40)	1 (1.33)	-	NH	-	-	NH	4 (15.83)	3.96	2	2.00
Eco RI	2 (21.00)	6* (10.23)	2 (4.71)	1 (10.31)	1 (1.28)	1 (11.50)	-	NH	13 (59.03)	4.54	6	2.17
Eco RV	-	2 (10.85)	2 (6.84)	-	-	1 (2.74)	3 (20.56)	-	8 (40.99)	5.12	4	2.00
Hin dIII	2† (5.26)	3† (2.79)	2 (3.65)	1 (1.25)	4 (12.23)	NH	1 (1.80)	NH	13 (26.98)	2.08	6	2.17
TOTALS	15 (39.37)	29 (47.83)	35 (56.17)	6 (21.57)	19 (32.51)	5 (17.39)	12 (31.19)	0 (0)	121 (246.13)	22.94	45	23.99

a Origin of sequences as determined in Section 4.3.5.5.5. (C= chloroplast, N= nuclear)

† more bands in some tracks

* more bands in longer exposure

\- this combination not tested

NH no detectable hybridisation

TABLE 25. Screening for somaclonal variation using RFLP analyses (II)

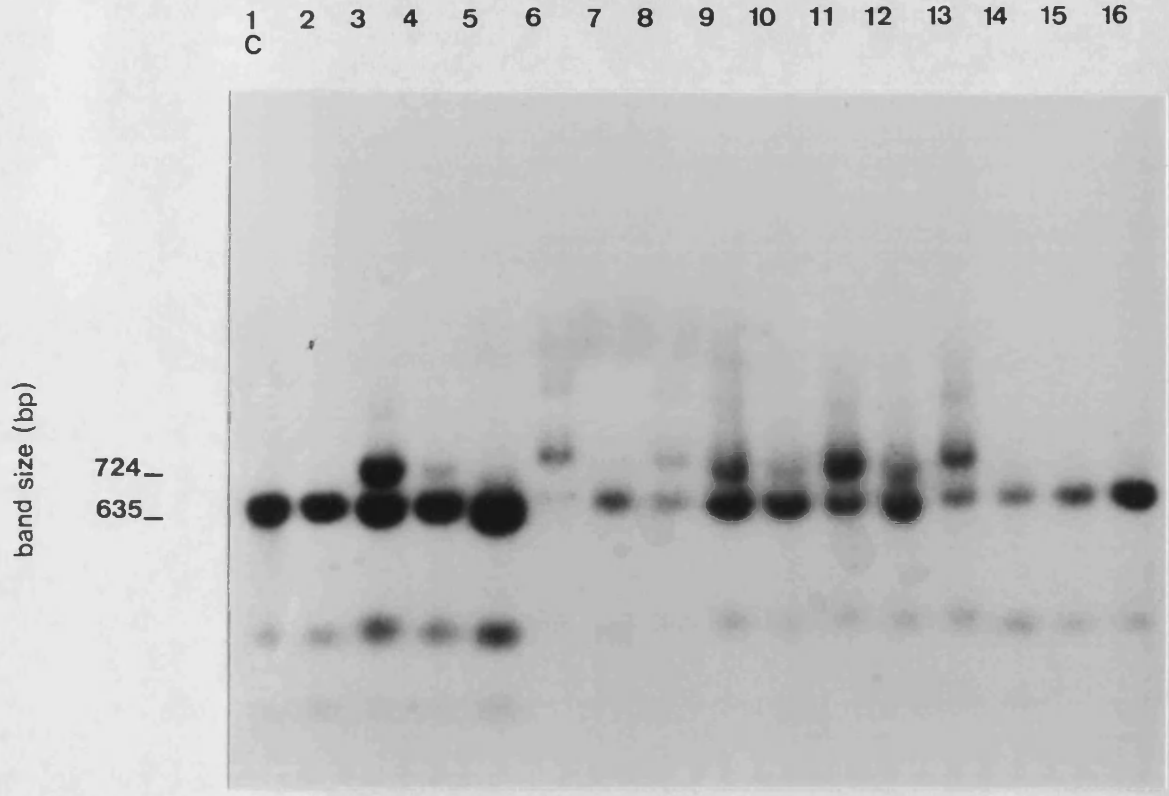
Batches of regenerated plants were screened for variation by hybridising genomic clones of varying copy number with DNA digested with restriction enzymes. RFLPs (R) and copy number variation (C) detected are indicated.

RESTRICTION ENZYME	PLANTS	PROBES								NO. OF GELS WITH DIFFERENCES	TOTAL NO. OF SUCCESSFUL GELS	% OF GELS WITH DIFFERENCES
		HIGH COPY NO.		MEDIUM COPY NO.	LOW COPY NO.							
		#58	#303	#444	#308	#442	#443	#526	#604			
Alu I	1-16	R? C?	C?	0	NH	0	0	0	NH	2 (0)	6	33.3 (0)
	50-65 I	-	C?	R? C?	-	-	0	0	-	2 (0)	4	50.0 (0)
	50-65 II	-	C?	0	-	-	-	-	-	1 (0)	2	50.0 (0)
Hae III	1-16	0	0	0	0	0	-	-	NH	0	5	0
Rsa I	1-16	R?	0	C	0	0	-	-	-	2 (1)	5	40 (20)
Taq I	1-16	0	-	0	0	0	-	-	NH	0	4	0
	1-23	-	R? C?	0	-	-	-	0	-	1 (0)	3	33.3 (0)
	50-65	-	C?	0	-	-	-	R?	-	2 (0)	3	66.7 (0)
Bam HI	1-16	0	0	0	0	0	-	-	NH	0	5	0
Bgl II	1-16	NH	0	0	-	NH	-	-	NH	0	2	0
Eco RI	1-16	0	C?	0	0	0	0	-	NH	1 (0)	6	16.7 (0)
Eco RV	50-65	-	0	0	-	-	0	0	-	0	4	0
Hin dIII	1-16	C?	C?	0	0	0	NH	0	NH	2 (0)	6	33.3 (0)
NO. OF GELS WITH DIFFERENCES		3 (0)	7 (0)	2 (1)	0	0	0	1 (0)	0	13 (1)		
TOTAL NO. OF SUCCESSFUL GELS		7	12	13	6	7	4	6	0		23.6 (1.8)	
% GELS WITH DIFFERENCES		42.9 (0)	58.3 (0)	15.4 (7.7)	0	0	0	16.7 (0)	0			23.6 (1.8)

R RFLP(s) detected
C copy number changes
? possible partial digestion (see text for discussion)
0 no variation
NH no hybridisation
\- combination not tested

Plate 23:

Recurrence of RFLPs in DNA from several plants regenerated from unrelated cultures and from control seed-grown plants (C), digested with *Alu* I (above) and *Taq* I (below) and probed with a 1.1kb, high copy number sequence (#303)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
C
band size (bp)
724
635

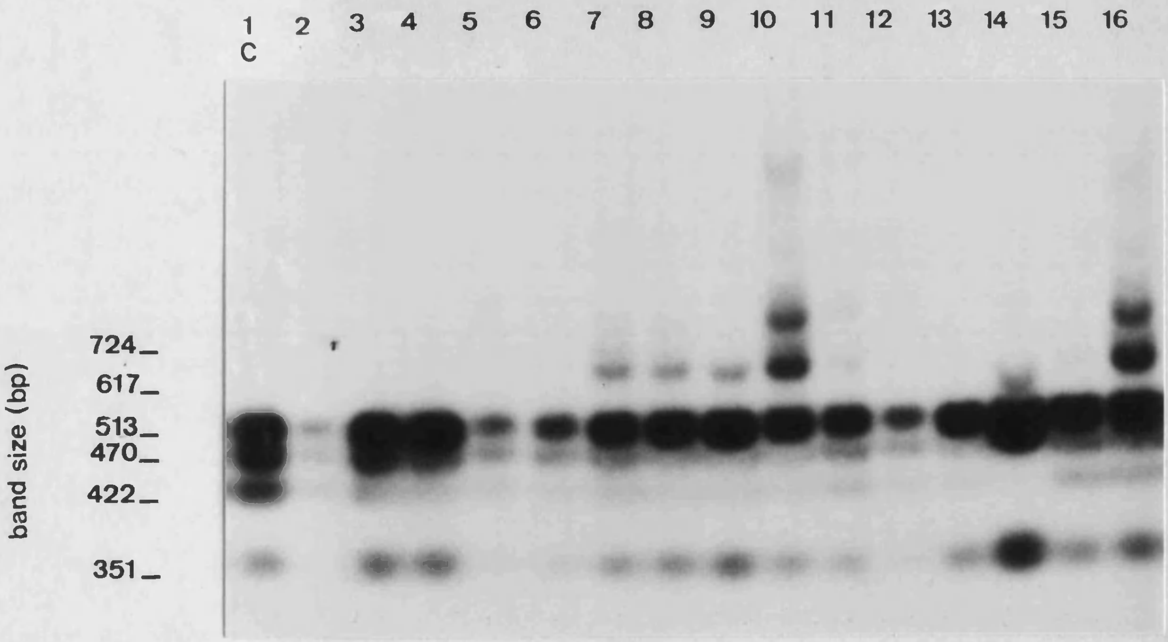
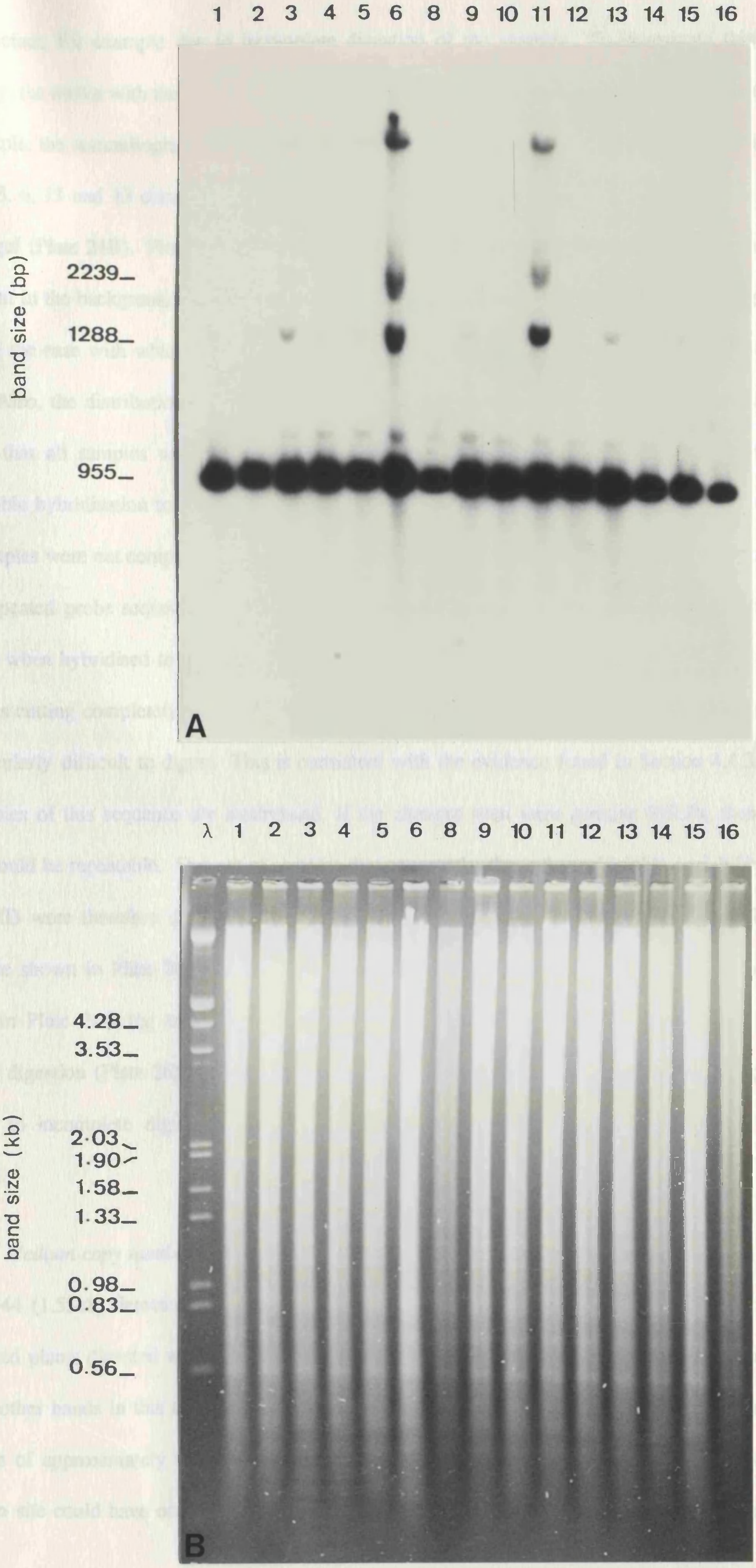

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
C
band size (bp)
724
617
513
470
422
351

Plate 24:

(A) *Hin* dIII digestion of the DNA from a control seed-grown plant (track 1) and from 14 regenerated plants, probed with a 1.1kb, high copy number sequence (#303)

(B) The original gel


1 2 3 4 5 6 8 9 10 11 12 13 14 15 16
band size (bp)
2239
1288
955
A
λ 1 2 3 4 5 6 8 9 10 11 12 13 14 15 16
band size (kb)
4.28
3.53
2·03
1.90
1.58
1.33
0.98
0.83
0.56
B

be an artefact, for example due to incomplete digestion of the samples. To investigate this latter possibility, the tracks with the bands in question were examined for evidence of incomplete digestion. For example, the autoradiograph in Plate 24A, showing apparent variation in copy number of bands in samples 3, 6, 11 and 13 compared to the control (track 1), was compared with a photograph of the original gel (Plate 24B). Firstly, it can be seen that intense bands, representing repeated sequences, are present in the background smear of each track on the gel; this is suggestive of complete digestion (although the ease with which the DNA cuts may vary throughout the genome, as shown in Section 4.4.3.3. Also, the distribution of DNA in each track appears to be similar (Plate 24B), which also suggests that all samples were completely digested. On the autoradiograph, however, there was considerable hybridisation to high molecular weight bands in tracks 6 and 11. This could indicate that these samples were not completely digested. It is notable that this problem was only found with the 2 highly repeated probe sequences (Table 25); other probes did not show any evidence of incomplete digestion when hybridised to *the same gel* (for example, see Plate 25). This suggests that most of the genome is cutting completely, but that the highly repeated probes are for regions of the genome that are particularly difficult to digest. This is consistent with the evidence found in Section 4.4.3.3 that some copies of this sequence are methylated. If the changes seen *were* genuine RFLPs, then these results would be repeatable. One set of samples that apparently showed considerable variability with probe #303 were therefore digested on two different occasions with the same enzyme, *Alu*I. The results are shown in Plate 26A and B. The upper, 724bp band, which is very prominent in some samples in Plate 26A (eg track 3), is much fainter (in comparison with the 635bp band) in the duplicate digestion (Plate 26B). This is strong evidence that the variability seen in this 724bp band was due to incomplete digestion. Possible causes of and remedies for this have already been discussed.

4.4.3.6.2 Medium copy number sequence

Probe #444 (1.55kb) detected one change in relative band intensity in the DNA of one out of 30 regenerated plants digested with *Rsa*I (Plate 27). In track 11, the 1.04kb band is considerably brighter than the other bands in this track, and the 0.86kb band is very faint; in all other tracks, the top four bands are of approximately equal intensity. I suggest two possible explanations for this: loss of a restriction site could have occurred, causing the replacement of 2 smaller bands with one larger one,

Plate 25:
The gel shown in Plate 24B, probed with a 1.25kb, low copy number sequence (#308)

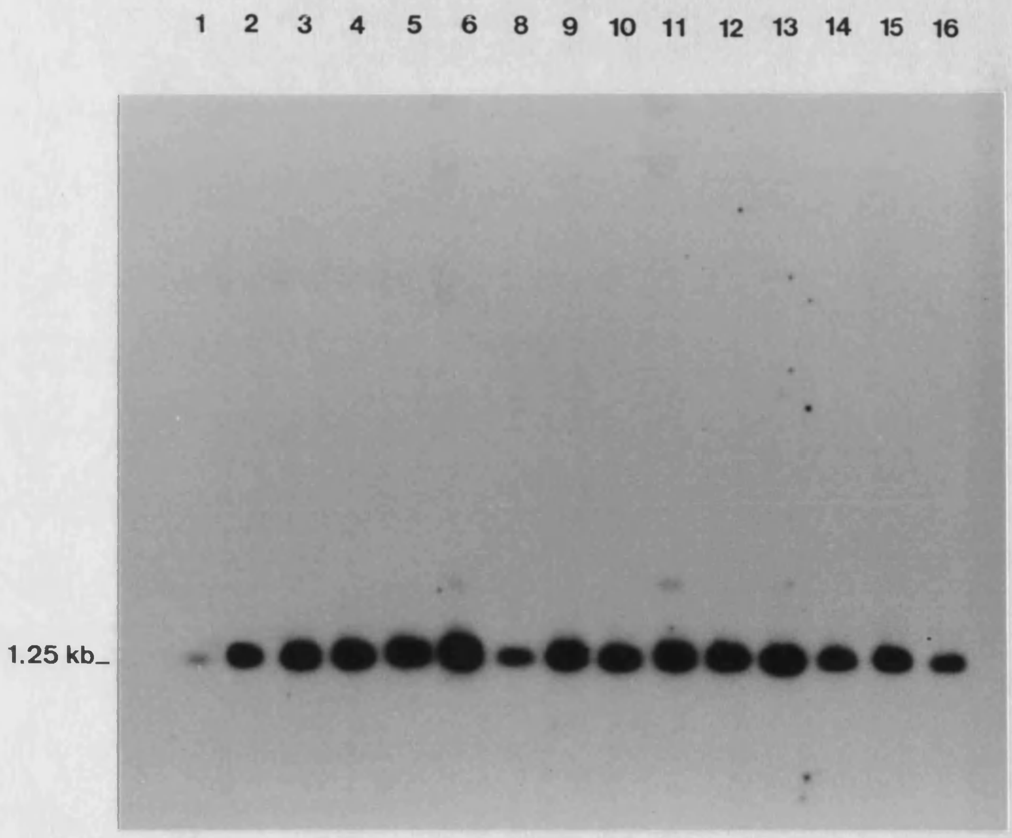
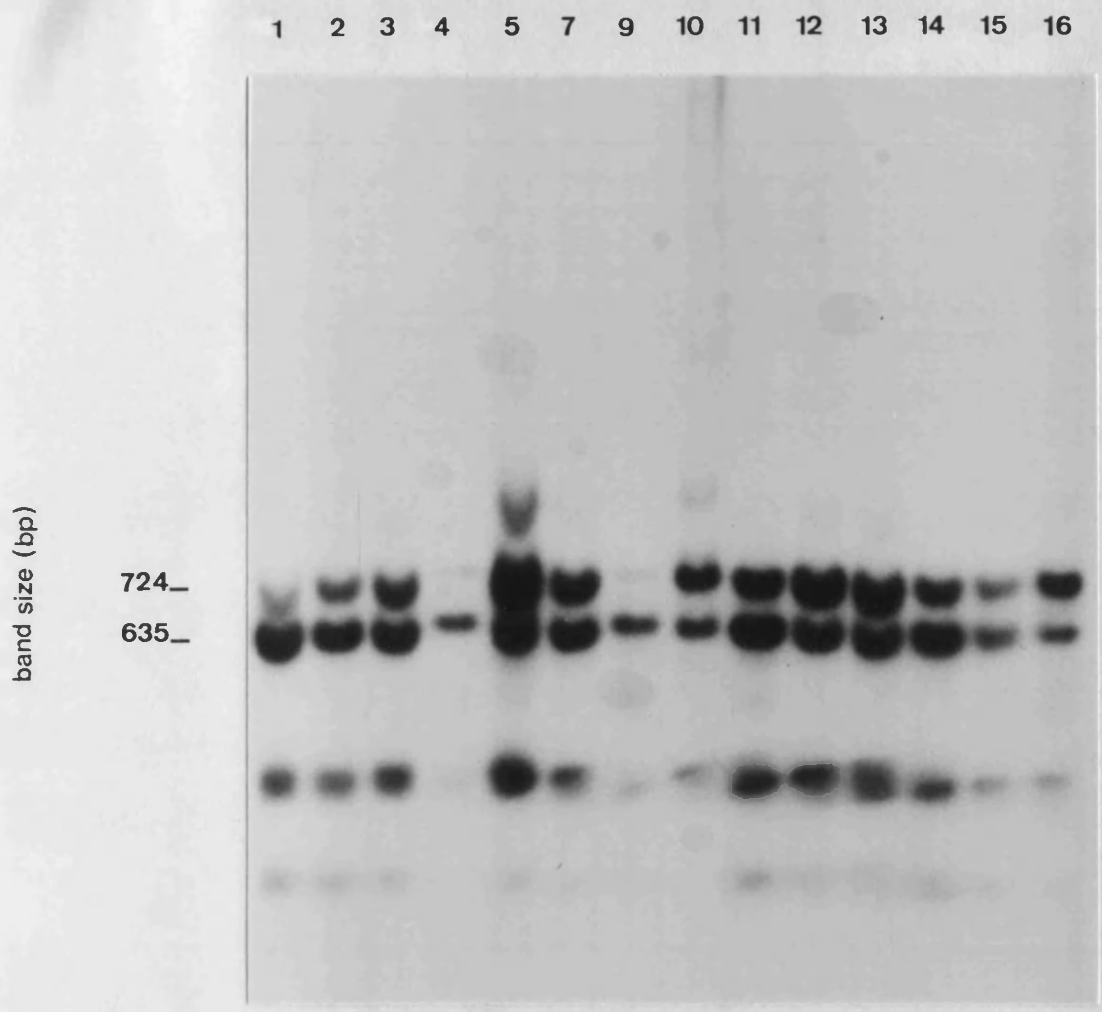

1 2 3 4 5 6 8 9 10 11 12 13 14 15 16
1.25 kb

Plate 26:

Duplicate *Alu* I digestions of the DNA of 1 control, seed-grown plant (track 1) and 15 regenerated plants, probed with a 1.1kb, high copy number sequence (#303).


1 2 3 4 5 7 9 10 11 12 13 14 15 16
band size (bp)
724
635

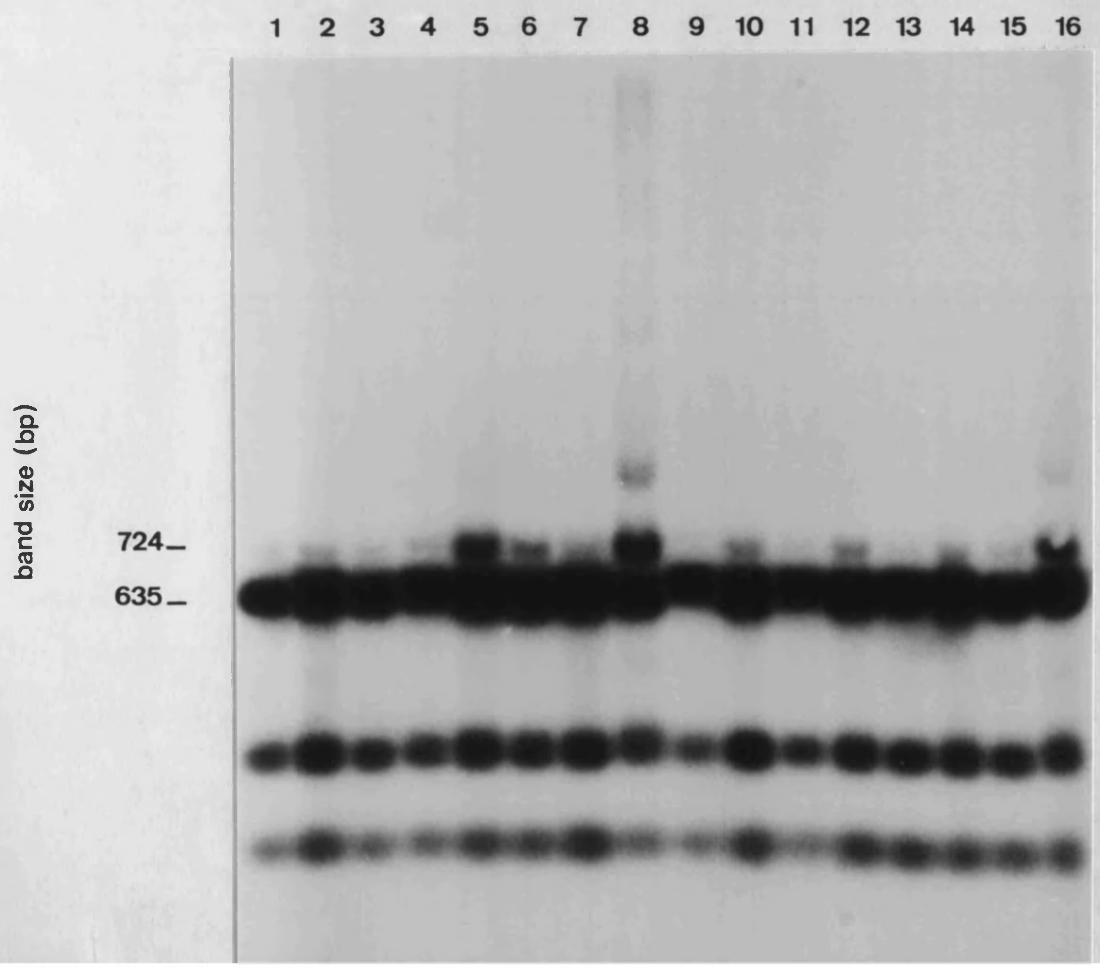

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
band size (bp)
724
635

Plate 27:
A *Rsa* I digestion of the DNA from a seed-grown, control plant (track 1) and from 15 regenerated plants, probed with a 1.55kb, medium copy number sequence (#444). An increase in the relative intensity of the 1.04kb band occurred in track 11.

or the change in relative band intensity could be due to a gain in the number of copies of the 1.1kb band, either by duplication or by the gain of a whole chromosome, or to a loss of the chromosome or chromosome fragment bearing the other copies of the sequence. In support of the latter hypothesis, this particular plant *did* have very few flowers, which showed partial incomplete fusion of the petals into the corolla tube; such a phenotype has been shown to be associated with high chromosome number (Section 3.2.1). However, if an alteration in chromosome number was responsible for the altered copy number observed, then other restriction enzymes should also have detected similar variation. From Table 25, it can be seen that no other restriction enzymes detected any variation in this probed region. It therefore seems unlikely that this hypothesis is correct. The distribution of this sequence throughout the genome and the chromosomal variation (if any) in the variant plant could be investigated further by *in situ* hybridisation of the probe to chromosome spreads.

The observation that *Rsa* I was the only restriction enzyme to reveal this variation supports the hypothesis that the underlying genetic change was a minor one, for example a point mutation, rather than a gross chromosomal change. This hypothesis could be tested by screening with enzymes which have sites that overlap with that of *Rsa* I , or ultimately by sequencing the probed region in this plant and a control plant. It would also be important to confirm the genetic nature of the variation by testing the inheritance of the RFLP.

The combination of *Alu* I and probe #444 produced a complex band pattern, with 7 major bands and several fainter ones. In the gel shown in Plate 28A, the intensity of one band (680bp, arrowed) varied between tracks; the band appeared to be absent in track 1 (a control, seed-grown plant). In a duplicate digestion, however, the 684bp fragment was only visible in a few tracks and was very faint (Plate 28B). A comparison of the two gels in Plate 28 shows that the intensity of the 684bp band was not the same in the two replicates of each track: I therefore suggest that this band is a product of incomplete digestion, and that it was broken down further in the more complete digestion.

This example highlights the importance of repetition in confirming a genetic basis for apparent restriction fragment length polymorphism.

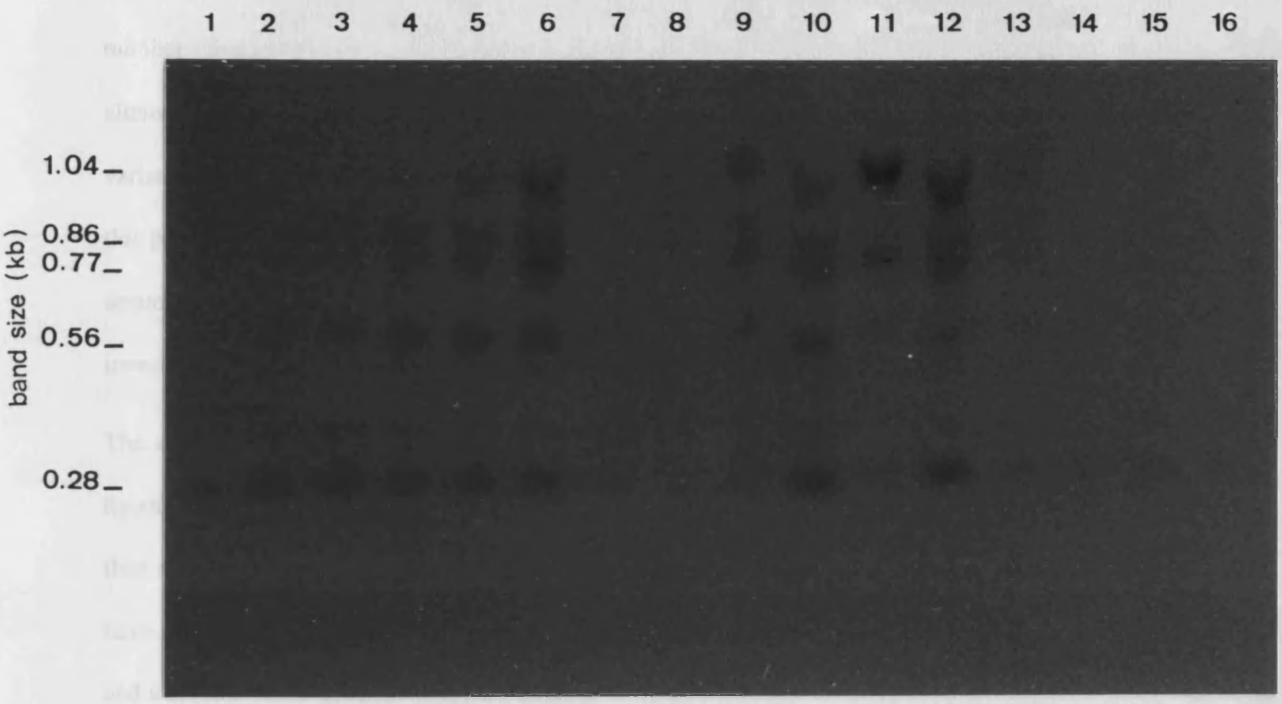
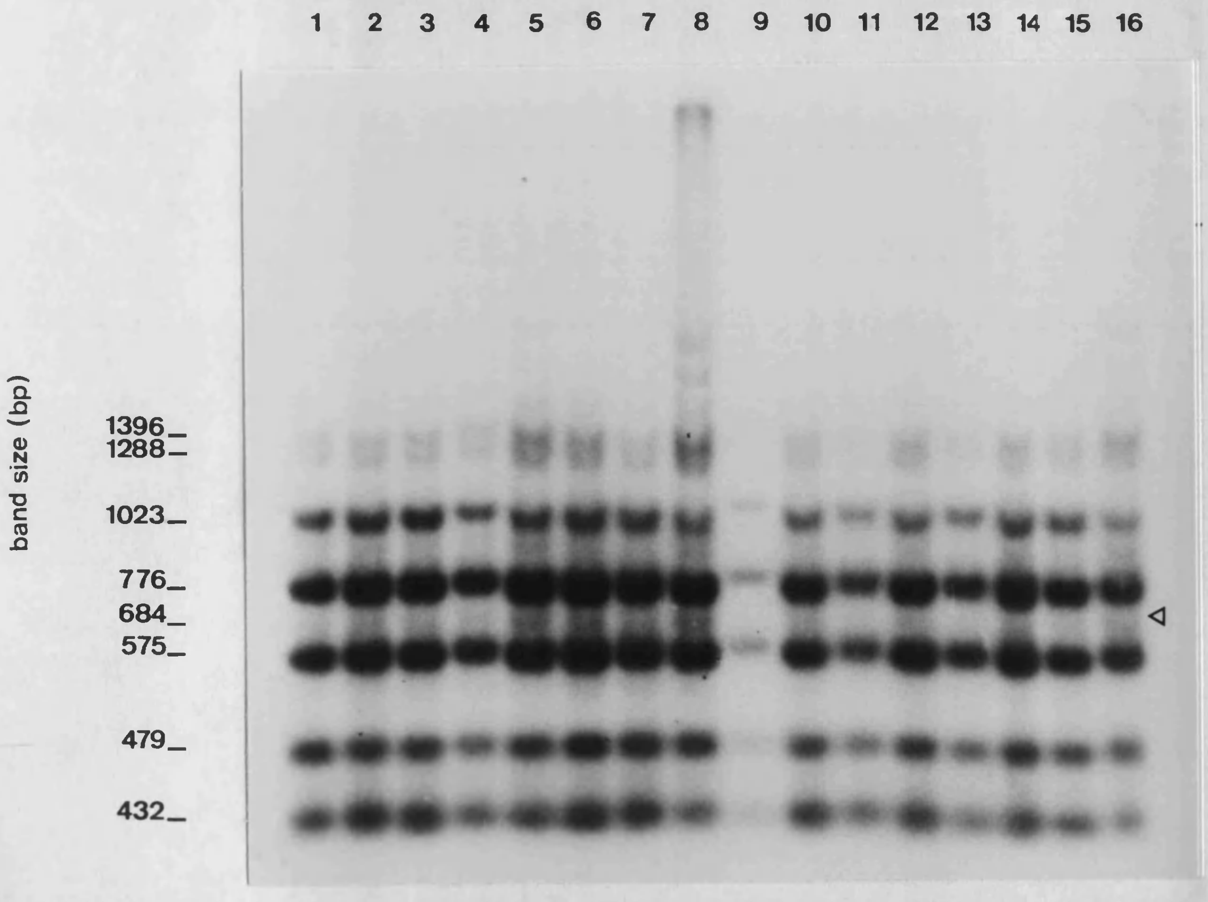

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
band size (kb)
1.04
0.86
0.77
0.56
0.28

Plate 28:

Duplicate *Alu* I digestions of the DNA from one control, seed-grown plant (track 1) and from 15 regenerated plants, probed with a 1.55kb, medium copy number sequence (#444).


1 2 3 4 6 7 9 10 11 12 13 14 15 16
band size (bp)
1396
1288
1023
902
776
684
575
479
432


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
band size (bp)
1396
1288
1023
776
684
575
479
432

4.4.3.6.3 Low copy number sequences

No variation was detected between regenerated plants using 5 low copy number sequences as probes, with the possible exception of probe #526 on a *Taq*I digest (Plate 29). The final 4 tracks each have a 650bp band that is not present in the other tracks. These four plants were regenerated from unrelated cultures. As it seems unlikely that an identical change would happen on four independent occasions, I suggest that these bands are artefacts due either to better hybridisation or to poorer washing in these tracks. The first possibility seems more likely since the filters were hybridised rolled up in a tube, whereas they were washed flat.

4.4.3.7 CONCLUSIONS

Use of RFLP analyses for varietal identification: A recent review of the use of RFLPs in varietal identification is given by Ainsworth and Sharp (1989). "Useful" levels of variability have been detected between varieties of maize (Helentjaris *et al.*, 1985), wheat (May and Appels, 1987), barley (Bunce *et al.*, 1986) and pea (Polans *et al.*, 1985), but in tomato, RFLPs could distinguish only between wild species, not between commercial varieties (Helentjaris *et al.*, 1985). In tobacco, RFLPs have been detected between species (Jamet *et al.*, 1987; Speeckaert and Jacobs, 1988). The results in Sections 4.4.3.1-4.4.3.3 show that RFLPs are readily detectable between different species and cultivars of *Nicotiana*. However, some cultivars had identical band patterns, so it would be necessary to use more than one probe to make precise identification possible. The higher degree of variability between tobacco cultivars than that reported between cultivars of the related Solanaceous species, tomato, may be due to the amphidiploid nature of *Nicotiana tabacum*.

Frequency of somaclonal variation detected using RFLPs: one of the 30 regenerated plants screened using RFLPs had an alteration in copy number (Table 25, Plate 27). Although other apparent differences were observed, it was concluded that these were artefacts due to incomplete digestion of some plant DNA samples.

In assessing the amount of variation that occurred, the total amount of the genome analysed should be taken into account. From Table 24, it can be seen that bands totalling 246.13kb were detected on autoradiographs; this estimate assumes, though, firstly that each visible band represents one copy of the sequence only, and secondly that none of the probed regions overlap. Since some of the data

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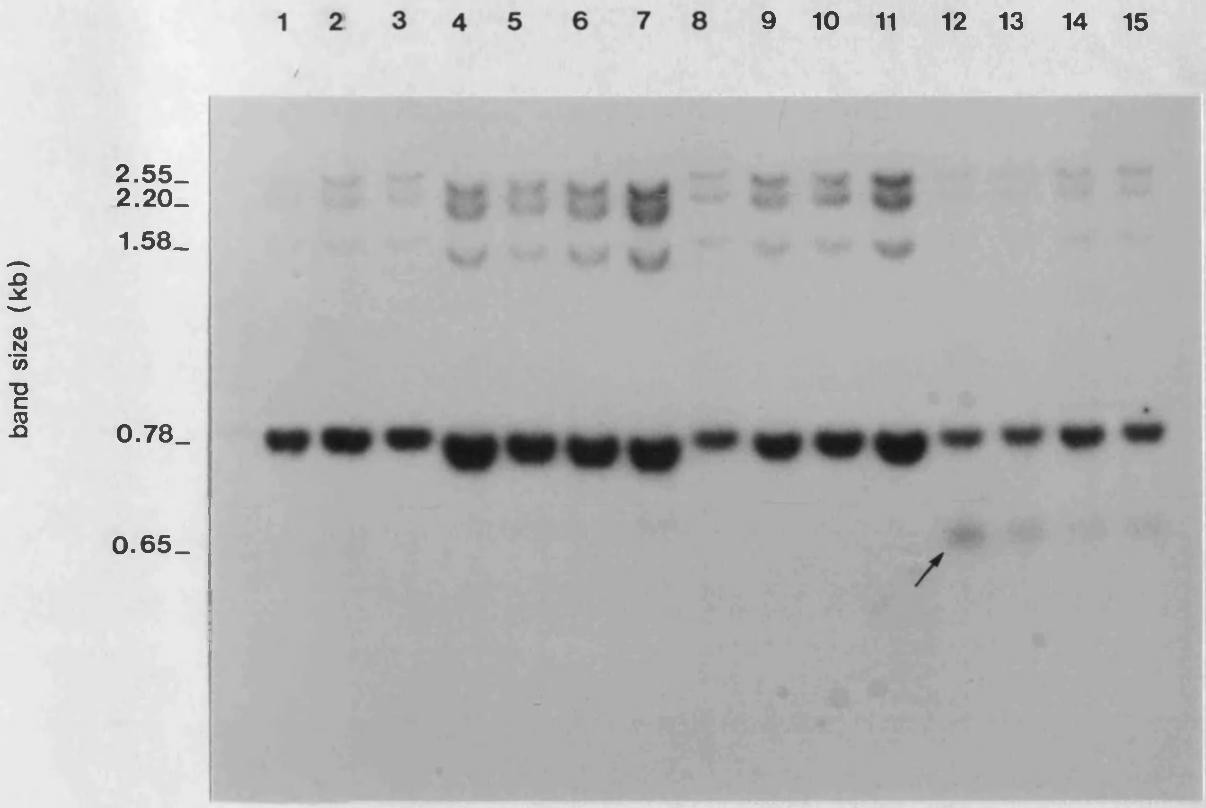
Plate 29:

A *Taq* I digestion of the DNA from a control, seed-grown plant (track 1) and fourteen regenerated plants, probed with a 1.71kb, low copy number sequence (#526). The same 0.65kb band is present in each of the last four tracks (arrowed).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
band size (kb)
2.55
2.20
1.58
0.78
0.65

obtained were from different digests *hybridised with the same probe*, there must have been considerable overlap of the regions screened in each case.

The total size of the *Nicotiana tabacum* genome has been estimated to be from 6.6pg per cell (Goldberg *et al.*, 1978) to 11.71pg per cell (Narayan, 1987); this is equivalent to 6.03×10^6 to 1.07×10^7kb. The fraction of the genome screened was thus tiny. If the region screened was "representative" of the whole genome, i.e. if the mutation rate was a reflection of that in the genome as a whole, then it can be seen that the overall number of mutations could have been considerable. The accuracy of an estimate of the frequency of somaclonal variation is likely to increase with an increase in the number of plants screened and in the total amount of the genome screened. It is, for this reason, difficult to make direct comparisons between my results and those of other researchers on tobacco. There are two published accounts of the use of RFLPs to study divergence of regenerated tobacco plants: Speeckaert and Jacobs (1988) found no variability of band patterns in 3 year-old protoplast-derived calli from *N. plumbaginifolia* using a moderately repeated sequence, while Grandbastien *et al.* (1989) selected spontaneous nitrate reductase deficient mutants from protoplast-derived calli and used a nitrate reductase cDNA clone as a probe to demonstrate RFLPs in one third of the mutants.

The lack of detection of variation in other sequences does not prove that no variation occurred. It has already been estimated that only a tiny fraction of the genome was analysed. The amount of variation detected may be unrepresentative of the whole genome, since the variability of the genome is not constant from one part to another. At one extreme, some coding sequences may be highly conserved and at the other, some sequences are the "hypervariable" sequences such as those discovered by Jeffreys *et al.* (1985). Even within a short sequence, some regions may be more prone to change than others: in a comparison of the base sequences of 12 copies of a maize satellite DNA sequence, Brown and Clegg (1983) found that mutable sites appeared to be clustered, and that C and G residues were significantly more prone to point mutations than A or T residues. This latter observation is in agreement with the results of Coulondre *et al.* (1978), which show that $^{5'}$ CG $^{3'}$ sequences are "hot-spots" for mutation. Even within the region screened, some mutations may have occurred without effect on the band patterns obtained. For example, in an *Alu*I digestion, the only point mutations that will give rise to a RFLP will be those that destroy an existing *Alu*I recognition site ($^{5'}$AGCT$^{3'}$) or create a new one.

To conclude, then. it is unwise to make inferences, from measurements of the variability in small parts of the genome, regarding the extent of variability throughout the whole genome. A much larger survey of different probes and restriction enzymes would be more likely to give a more representative picture of the extent of somaclonal variation.

Increasing the amount of the genome screened: to increase the total amount of the genome screened at once, several probes of the same copy number could be hybridised to one filter at the same time; if any variation was detected, the probes could be split into smaller groups to determine which had varied. Alternatively, a "DNA fingerprinting" system could be used. It has been shown that DNA from the bacteriophage M13 can be used to distinguish individual plants of some species (e.g. *Populus deltoides*— Rogstad *et al.*, (1988). Other species are more uniform, though. In human DNA, fingerprints have also been detected using synthetic oligonucleotide probes (Ali and Wallace, 1988). Such an approach could also be applied to plant DNA, for example using the sequence GAAGAA/G, which is known to be amplified in wheat (Flavell, 1986).

Factors affecting the success of RFLP analyses: Some guidelines concerning the choice of restriction enzymes and probes can be proposed from these results. Much of the tobacco genome appears to be methylated (Section 4.4.3.3), and would therefore be inaccessible to restriction enzymes that were sensitive to methylation. The use of such enzymes should therefore be avoided. A comprehensive listing of the known methylation sensitivities of restriction enzymes is given by Nelson and McClelland (1989).

No one restriction enzyme emerged as superior to others in the detection of RFLPs. Not enough RFLPs were detected among regenerated plants to enable a statistical comparison of different enzymes to be carried out; for screening cultivars, there was no statistically significant difference between enzymes with 4bp recognition sequences and those with 6bp recognition sequences (Section 4.4.3.3). These findings do not conform with the predictions of Wijsman (1984) that enzymes with 4bp recognition sequences would be the most efficient for the detection of point mutations. It is possible that the advantage of such enzymes is counterbalanced by the loss of information due to the indetectability of very small fragments. However, the minimum detectable fragment size in these experiments was about 200bp; at this level, enzymes with 4bp recognition sequences are still thought to be more efficient for detecting RFLPs (Wijsman, 1984). This loss of information with the loss of small fragments may be a particular problem when using restriction enzymes that have extremely frequent recognition sequences; for example, the *Nicotiana tabacum* chloroplast genome is cut by *Taq*I into 789 fragments, of which 502 are less than 200bp long (information obtained from an analysis of the complete nucleotide sequence of the chloroplast genome (see Section 4.4.3.3). One way of improving the detection of such small fragments might be to adjust DNA solutions to 0.01M Mg^{2+} prior to ethanol precipitation: this procedure is supposed to improve the efficiency of the precipitation of DNA molecules less than 200bp long (Maniatis *et al.*, 1982).

In Section 4.4.3.3, it was shown that there were statistically significant differences between the abilities of sequences of different different copy numbers to detect RFLPs between cultivars. Single copy sequences were significantly more likely to detect variation than were repeated sequences, even though it is thought that single copy, coding sequences are highly conserved. It may be that the introns, and the sequences flanking the transcribed region, are particularly prone to mutation. Similar conclusions were reached by Landry *et al.* (1987) and by Sharp *et al.* (1988). Random genomic clones are not necessarily composed of only one type of sequence class. For example, in tobacco, the single copy DNA is extensively interspersed with moderately repeated sequences (Zimmerman and Goldberg, 1977). It could be variation in these adjacent sequences that has been detected, rather than in the single copy regions. However, if a single copy probe contained a long stretch of repeated DNA, it would have been classed as a repeated sequence by colony hybridisation and dot blotting.

4.4.4 Summary

A genomic library of *Nicotiana tabacum* was constructed using the vector pUC13. The lengths and copy numbers of the clones were determined.

The potential application of RFLP analyses to the detection of genetic variation was investigated by screening plants with different levels of expected genetic heterogeneity. RFLPs successfully distinguished between different species of *Nicotiana*; under the conditions used, there was no hybridisation of the probe to the DNA of other Solanaceous species. The relatively low level of band sharing between different *Nicotiana* species makes this particular probe/enzyme combination an unsuitable one for a detailed analysis of the taxonomy of the genus.

The degree of uniformity was greater between cultivars of *Nicotiana tabacum* than between species of *Nicotiana*. Some probe/enzyme combinations could not distinguish between the cultivars tested. Low copy number sequences detected variation between cultivars significantly more frequently than higher copy number sequences. There was no significant difference, in abilities to detect RFLPs between cultivars, between enzymes with 4 or 6bp recognition sequences. Deductions from band patterns concerning the relationships of the different cultivars tested were consistent with the known origin of these cultivars.

No RFLPs were found between individual plants from a seed-grown population or between different tissues of an individual plant.

61 restriction enzyme/probe combinations were screened for their abilities to detect RFLPs between 28 plants regenerated from tissue culture. Of these, 12 combinations (11 involving single-copy sequences) did not show any hybridisation. Of the remainder, one combination, *Rsa*I and a medium copy number sequence (#444) detected copy number variation in one regenerated plant.

In several experiments, but particularly with regenerated plants, there appeared to be RFLPs. The frequent occurrence of particular variants, however, and the disappearance of the variation on repetition of the experiments, led to the hypothesis that incomplete restriction digestion was the cause of the apparent variation. It was shown that, in repeated sequences, there was a high degree of methylation, which may have been the cause of some of the inhibition of digestion. Non-methylated sequences, such as chloroplast DNA, were readily digested to completion. These results indicate the importance of choice of restriction enzyme and sequence type in obtaining meaningful and reproducible data.

5. Epilogue

In Sections 3.3.1 and 3.3.2, it was shown that there was extensive chromosome number variation among plants regenerated from tobacco tissue culture. Of the 30 regenerated plants screened for RFLPs, one was aneuploid and one mixoploid, and a further eight were classed, by their morphology, as having high chromosome number. Despite this high frequency of chromosomal abnormality, only in one of these plants was a RFLP observed (Section 4.4.3.6.2). Why was the chromosomal variation not detected more often using molecular biology? Any multiplication of whole sets of chromosomes would not change the relative intensities of bands, and would not, therefore, be detectable using RFLP analyses. However, my results and those of Sacristán and Lutz (1970) and Nuti Ronchi *et al.* (1981) have shown that many regenerated tobacco plants are *aneuploid* at the diploid or tetraploid levels, rather than being euploid; such variation should have been detectable using RFLP analyses. It is possible that, by chance, probes were never used for the particular chromosomes that were missing. An alternative explanation could be that because tobacco is amphidiploid, it therefore has at least four copies of many sequences, and the reduction in intensity of a band from 4 copies to 3 copies might not be detectable, particularly if the autoradiography film is not pre-flashed or where the film is already saturated and therefore cannot respond to the amount of radioactivity present in a linear fashion.

Both cytogenetic and molecular genetic techniques have their place in the analysis of somaclonal variation; in some cases, it may be important to know the chromosomal status of a plant in order to explain the variation detected using molecular biology. It would be interesting to combine these two techniques in the investigation of somaclonal variation by the *in situ* hybridisation of probes to chromosome spreads. Such an approach has been used to demonstrate deletions and translocations in repeated sequences in regenerated wheat×rye hybrid plants (Lapitan *et al.* (1988)); Huang *et al.* (1988) have used it to localise the single-copy parsley chalcone synthase gene, so, in theory, variation in single copy sequences could also be monitored in this way.

If somaclonal variation is a relatively rare phenomenon, then large-scale screening procedures will be necessary to detect it. RFLP analysis is costly and time-consuming (Beckmann and Soller, 1983; Ainsworth and Sharp, 1989) and so may be impractical for such large-scale screening. In order to screen very large numbers of somaclones, a more efficient method of detecting variation might be to choose a gene with a readily measurable phenotypic effect, and to pre-screen regenerated plants for variation in this phenotype. Molecular genetic analyses could then be used to investigate the nature of the variation. However, as discussed in Section 1.2.1, much genetic variation may have no obvious effect on the phenotype and may therefore escape detection in the pre-screening stage.

This work has demonstrated that high levels of chromosome number variability occur in regenerated plants; such variability may lead to sterility and to genetic instability. This could be a severe limitation of the application of somaclonal variation to the creation of new plant varieties. The prospects for eliminating chromosomal variability by manipulation of tissue culture conditions would not seem good in the light of the finding that much of the variation is present in the explant itself; a more productive line of research might be an investigation of the effects of the origin and age of the explant on genetic stability *in vitro*.

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Appendix 1: tissue culture media

Murashige and Skoog medium (MS)

COMPONENT	*MOLARITY*
NH_4NO_3	*20.61mM*
KNO_3	*18.79mM*
$CaCl_2.2H_2O$	*3.00mM*
$MgSO_4.7H_2O$	*3.07mM*
KH_2PO_4	*1.25mM*
H_3BO_3	*0.10mM*
$MnSO_4.H_2O$	*0.13mM*
$ZnSO_4.4H_2O$	*37.00µm*
$NaMoO_4.2H_2O$	*1.03µm*
$CuSO_4.5H_2O$	*0.10µm*
$CoCl_2.6H_2O$	*0.11µm*
Ferric EDTA	*0.10mM*
KI	*5.00mM*
Inositol	*0.56mM*
Nicotinic acid	*4.06µm*
Pyridoxine.HCl	*2.43µm*
Thiamine.HCl	*2.96µm*

pH 5.6

Cereal protoplast wash solution (CPW)

COMPONENT	*MOLARITY*
KH_2PO_4	*0.2mM*
KNO_3	*1.0mM*
$CaCl_2.2H_2O$	*10.0mM*
$MgSO_4.7H_2O$	*1.0mM*
KI	*1.0µm*
$CuSO_4.5H_2O$	*0.1µm*

pH 5.8

Appendix 2: preparation of stains

(Protocols from Evans and Reed (1981))

Carbol fuchsin:

3g of basic fuchsin (Gurr) were added to 100ml 70% ethanol to give Solution A. 10ml Solution A were added to 90ml of 5% (v/v) phenol in distilled water, giving Solution B. 45ml Solution B were added to a mixture of 6ml glacial acetic acid and 6ml of 37% (v/v) formaldehyde, to give Solution C. To make the working solution, 5ml Solution C were added to a 95ml mixture of 1.8g sorbitol in 45% (v/v) acetic acid (*Note*: the sorbitol was dissolved in water before adding acetic acid). The stain was aged for at least 2 weeks before use, and was stored at room temperature.

Feulgen:

1g of basic fuchsin and 1.9g potassium metabisulphite were added to 100ml of 0.15N HCl, and left for 24 hours. 0.5g activated charcoal was added to the mixture, which was then filtered through Whatman #1 filter paper pre-moistened with 1N HCl. The filtration step was repeated until the stain was completely colurless. The stain was stored in the dark at 4°C.

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